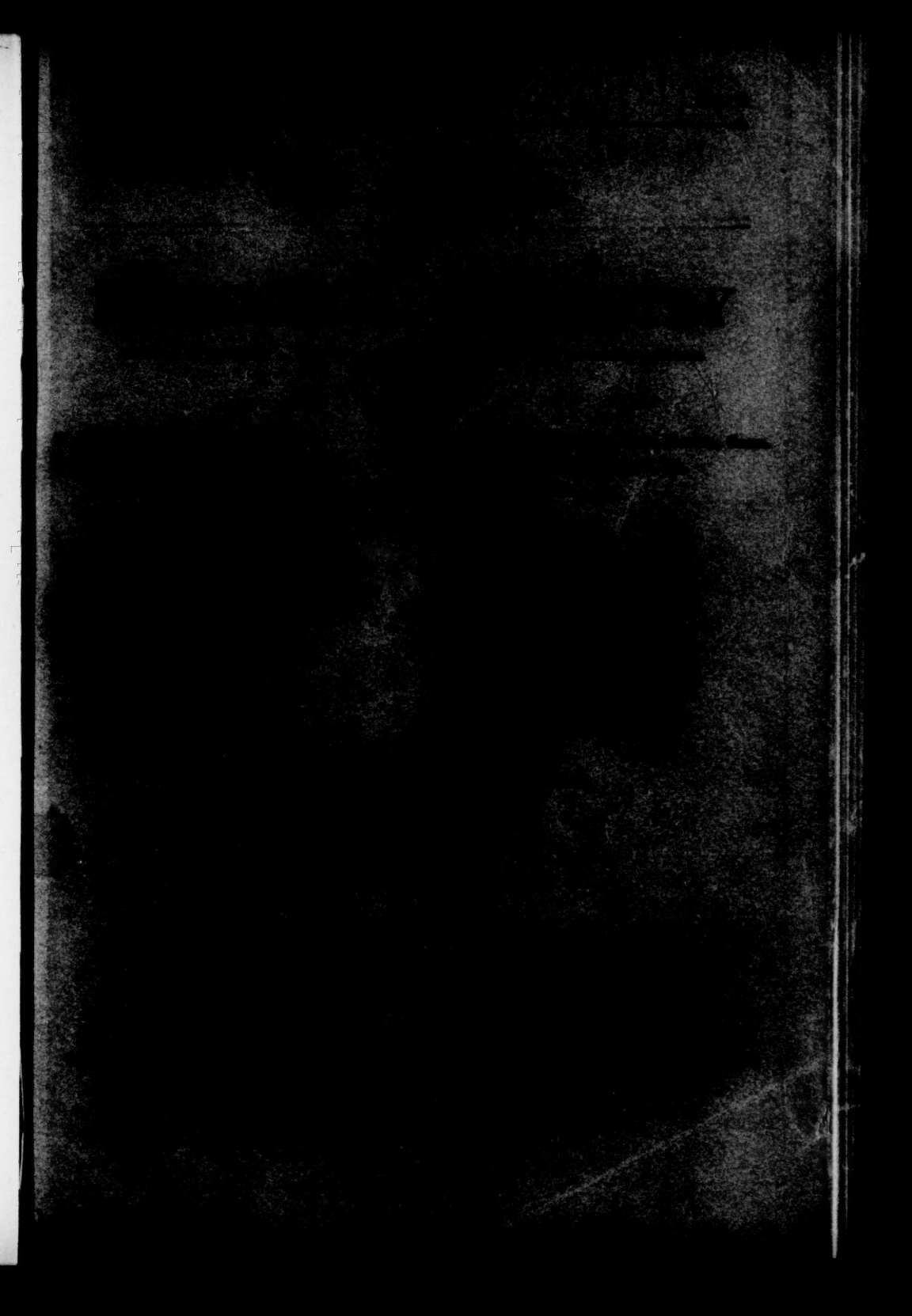

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NUTRITIVE VALUE OF THE PROTEINS OF THE BARLEY, OAT, RYE, AND WHEAT KERNELS.*

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Investigators are at present in accord in the conviction that none of the common cereal grains alone or even a mixture of them suffices to afford satisfactory nutrition. The reason for this is to be found primarily in a deficiency in certain of the essential dietary components, notably specific inorganic elements and fat-soluble vitamine. The inadequacy with respect to such needed factors may be complete or partial; in either event nutrition, especially during growth, will be impaired. The particular deficiencies just referred to can readily be offset by supplying otherwise the items needed. The foremost remaining dietary factor in these seeds is the protein. In some of them the proportion of protein calories—the nutritive ratio—is rather low. Furthermore, it has been demonstrated conclusively that some of the individual proteins, like zein (maize), gliadin (wheat), and hordein (barley), for example, are chemically defective and, correspondingly, physiologically inadequate proteins. Hence a misconception of the possible value of the cereals as sources of protein has developed in the minds of some persons owing to their failure to realize that in the form in which these grains are most commonly fed the sum total of their various proteins must be taken into account.

*The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

In attempting a further study of the relative nutritive value of the cereal proteins we have already expressed the fundamental view-point as follows:

"The comparison of the cereals, which resemble each other closely in being composed in major part of starch and other carbohydrates and are somewhat alike in respect to the presence or absence of different inorganic nutrients and vitamines, rests in a large measure upon the relative equivalence of their unlike proteins for the uses of nutrition. An ideal comparison, from this standpoint, requires a diet adequate and equivalent in respect to all other essentials, both organic and inorganic, known to constitute a perfect food when suitable protein is supplied therewith."[1]

For the white rat which we have employed in these investigations it is essential to have a considerable concentration of protein in the diet if suitable growth is to be attained. This is particularly true when the quality of the protein is somewhat inferior so that relatively large quantities are needed to furnish the indispensable minimum of the limiting amino-acids. Furthermore, if any considerable proportion of other foods is added to furnish the lacking indispensable non-protein factors, the protein content of the resulting dietary mixture becomes correspondingly diluted.

With these facts in mind we attempted to prepare protein concentrates from various cereal grains by removing as much as possible of the starch of the seeds. The preliminary results obtained with such concentrates, supposedly representing the bulk of the total protein in the respective cereals, were not entirely satisfactory.[1] There was an unlike inclusion of indigestible residues—presumably carbohydrates—in the different products; so that these inevitable conditions rendered accurate comparisons on a protein basis somewhat inconclusive.

The concentrates used represented only about 80 per cent of the total proteins of the seed. At that time we thought that in order to get the accurate food intakes which alone permit the drawing of definite conclusions regarding the relative nutritive values of the different proteins, it was necessary to use a food mixture rich in fat. Inasmuch as the cereals are low in protein, the addition of sufficient fat to make the food of the proper consistency increased its calorific value so greatly that too little

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 521.

protein was eaten to promote satisfactory growth. In our investigation of the nutritive value of wheat[2] we found that it was possible to obtain accurate food intakes on diets containing as little as 5 per cent of fat. This was done by adding to the mixture of finely ground wheat, salts, and butter fat enough water to make a dough which the rats would not scatter. This food contained only about 3.75 calories per gm. as compared with our ordinary fat-rich foods which contain on the average about 5 calories per gm. Consequently the animals ate much more liberal quantities of the 5 per cent butter fat food than they did of the fat-rich food, so that although the *percentage* of protein in the former food might be relatively low, the *total* consumption of protein on this food was as great as that on the fat-rich, protein-rich foods ordinarily used. This method, therefore, made it possible for us to study the unchanged total proteins of the various seeds at different protein levels and compare their nutritive value according to the numerical method which we have previously described.[3]

We have resumed the investigations by feeding the entire cereal grains, finely ground, along with an adequate salt mixture and sufficient butter fat to supply the fat-soluble vitamine. We have assumed, on the basis of evidence obtained by both ourselves and others, that the quantities of the entire cereal grains used by us would supply sufficient water-soluble vitamine.

The method employed in this series of comparative experiments was to feed the finely ground seed mixed with 3 per cent of a suitable salt mixture, 5 per cent of butter fat, and, where necessary, enough corn-starch to make the total protein of the ration 5, 8, or 10 per cent, approximately. In this way the proteins of barley, oats, rye, and wheat were compared.

Each day a little more than enough of the finely ground food for one day's feeding was mixed with a quantity of water sufficient to make a soft dough, and the mixture was packed into the food cups. The next morning the food that remained was dried in an oven. At the end of the week the residues were collected, weighed, and their weight was deducted from that of the dry

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

[3] Osborne, T. B., Mendel, L. B., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxvii, 223.

ingredients fed. Care was taken to make the daily excess of food so small that inaccuracies involved in drying the residues were reduced to a minimum.

Barley.

In preparing the foods intended for the study of the nutritive value of the barley proteins special care was taken to grind the grain, including the husks or glumes, to a very fine powder. The precise composition of the various food mixtures used was as follows:

	10 per cent protein food.	8 per cent protein food.	5 per cent protein food.
	per cent	*per cent*	*per cent*
Ground barley	92	72.0–75.5	45–47
Salt mixture*	3	3.0	3
Butter fat	5	5.0	5
Starch	0	20.0–16.5	47–45

*The composition of the salt mixture employed is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

The results of the feeding experiments with these mixtures, as exhibited in the gains of body weight of growing rats, are shown in Charts I, II, and III.

These experiments leave no doubt of the adequacy of the barley *proteins* as a whole in the nutrition of growth; for several of the rats grew to large adult size without any other source of protein than that derived from this cereal. Some of the animals on the higher percentages of barley protein even surpassed the normal rate of growth on ordinary mixed food. With the lower concentration of barley in the food the deficit of protein appears as a factor limiting the rate of growth, though even on the lowest percentage not inconsiderable gains were made. The possibility of growth upon a diet in which barley supplied the protein was earlier indicated in our experiments with barley concentrates.[1] These tests are less conclusive than the present series because the rations included, during part of the period, a small amount of brewer's yeast containing protein which possibly supplemented the barley protein so as to increase the rate of growth.

In a valuable contribution on the dietary qualities of barley, Steenbock, Kent, and Gross[4] have reached the conclusion that

"The protein content of barley (13.6 per cent) is too low for continued growth at the normal rate. When reduced in amount to 8.1 per cent, the small amount of growth that results is soon followed by a decline. 5.4 per cent just about suffices for maintenance.

The primary growth determinant in barley is inorganic salts. Of secondary importance, but no less urgent, are protein and fat-soluble vitamine."

Steenbock, Kent, and Gross[4] have reproduced the growth curve of one rat (No. 25, Chart 10) which made excellent gains on barley, unsupplemented by other protein. Why their other rats on the same diet did not grow so well is not clear. Our animals ate the rations in liberal amounts, as the food intakes which we have measured indicate.

The composite growth curve published by McCollum, Simmonds, and Parsons[5] for rats on a diet in which all the protein, amounting to 9 per cent of the ration, was derived from barley also fails to indicate a degree of gain comparable with that attained by our animals on rations containing 8 per cent of barley proteins. It should be noted, however, that our food mixture was not like that of McCollum, Simmonds, and Parsons which contained "seed = to 9 per cent protein; NaCl, 1.0; $CaCO_3$,1.5; butter fat, 3.0; dextrin to 100."

We have not made observations on the total length of life of animals which have grown up upon a diet furnishing proteins from a single seed like barley, or upon their ability to produce and rear young. We believe that there are too many other nutritive factors involved in successful nutrition to enable us, upon the basis of our present knowledge, to charge any failures of nutrition in the second generation to chemical inadequacy of protein solely, although it is quite conceivable that the level of protein metabolism represented by a relatively high or low content of protein in the ration over a long span of life may have a pronounced effect upon the maternal functions. It seems to us,

[4] Steenbock, H., Kent, H. E., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxv, 61.

[5] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 155, see Chart I, p. 164.

however, that if an animal is able to attain adult size upon a diet which furnishes protein from a single source, the nutritive value of this protein is clearly established. Our experiments with barley have demonstrated the possibility of attaining large size where proteins from this source alone were available—provided the total intake of food and consequently of protein and energy was adequate. In view of such positive results, in contrast with the less successful growth obtained in experiments made by others and conducted perhaps under less favorable conditions, it seems hazardous to venture a strict comparison of the nutritional value of proteins from the records of different investigators without more precise information of a quantitative nature regarding the relations of body gain to protein intake where the energy and other essential factors are strictly comparable.

In explanation of the less satisfactory growth observed on the diets containing the lower concentration of barley proteins it might be assumed that a deficiency of water-soluble vitamine, due to the smaller content of the seed in the ration, was the underlying cause. Judging from the evidence secured by Steenbock, Kent, and Gross[4] regarding the content of water-soluble vitamine in barley, it is not probable that a lack of the latter was the cause of poorer growth in our experiments with diets containing only 5 per cent of barley protein. The rats on the 5 per cent barley protein diets ate as much total food, *i.e.* calories, as those of the same size on the higher concentrations of protein. The quantities probably were as large as the animals could consume. Hence we conclude that the deficiency of protein was the direct outcome of its low content in the ration. We have, however, directed new experiments to the solution of this problem by furnishing a ration low in barley protein but unquestionably adequate in water-soluble vitamine. The diet was the same as in the earlier series, with the addition of approximately 18.6 mg. daily, apart from the food, of a protein-free vitamine preparation from yeast.[6] This quantity, which supplemented the water-soluble vitamine in the barley already present in the 5 per cent protein food, had been shown in test experiments to be adequate for promoting food intake and growth on our standard food

[6] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.

mixtures. As will be apparent from Chart XIII, recording these experiments, no nutritive advantage accrued from this vitamine

TABLE I.

Barley.

Protein in food.	Rat.	Initial weight.	4 week period.			10 week period.		
			Gain in weight.	Total food intake.	Gain per gm. protein eaten.	Gain in weight.	Total food intake.	Gain per gm. protein eaten.
per cent		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
10	5717 ♂	71	69	404	1.7	129	973	1.3
10	5716 ♂	64	64	376	1.7			
10	5701 ♂	72	63	334	1.9	120	950	1.3
10	5251 ♂	66	50	320	1.6	74	764	1.0
10	5709 ♂	70	49	340	1.4	117	983*	1.2
10	5256 ♂	70	43	352	1.2	92	829	1.1
10	5267 ♂	68	37	263	1.4	78	744	1.0
8	5406 ♂	58	78	397	2.5	187	1,178	2.0
8	5407 ♂	74	58	391*	1.9	122	1,090*	1.4
8	5654 ♂	85	57	364	2.0			
8	5649 ♂	71	55	408	1.7			
8	5659 ♂	66	46	297	1.9	114	917	1.6
8	5410 ♂	58	42	328	1.6	86	745*	1.4
8	5662 ♂	76	40	312	1.6	112	964	1.5
5	5427 ♂	74	43	423	2.0	84	1,043	1.6
5	5437 ♂	75	35	337*	2.0	49	807*	1.2
5	5650 ♂	75	33	406	1.6	79	1,091	1.4
5	5432 ♂	69	31	347	1.8			
5	5707 ♂	70	26	311	1.7	64	861	1.5
5	5656 ♂	75	23	316	1.5	49	871*	1.1
5	5661 ♂	67	19	241	1.6	47	645	1.5
5	5423 ♂	66	17	228	1.5	51	630	1.6
			Barley + Vitamine.					
5	6211 ♂	70	11	234	0.9			
5	6248 ♂	70	20	328	1.2			

* The records for the food intakes in these experiments were unsatisfactory.

supplement. The food intakes (Rats 6211 and 6248) showed no noteworthy increase; nor was the gain per gm. of protein eaten more favorable (see Table I), hence we must still ascribe the slow

growth on the 5 per cent barley protein ration to the comparatively small quantity of protein afforded by the diet.

Pearled Barley.—We have undertaken a few feeding experiments with pearled barley. This preparation differed from the entire barley kernel in lacking the husks or glumes and the greater part of the outer coats of the seed; *i.e.*, the bran. The food mixture consisted of

	per cent
Pearled barley	92
Salt mixture*	3
Butter fat	5

*The composition of the salt mixture employed is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

These ingredients were mixed with a little water and baked in an oven.

TABLE II.

Pearled Barley.

Protein in food.	Rat.	Initial weight.	4 week period.		
			Gain in weight.	Total food intake.	Gain per gm. protein eaten.
per cent		*gm.*	*gm.*	*gm.*	*gm.*
8	5005 ♂	83	34	302	1.4
8	4961 ♂	76	24	270	1.1
8	5010 ♂	67	13	237	0.7

The outcome of the experiment is shown in Chart IV and Table II. The growth obtained was relatively slight in comparison with the more adequate gains on foods of a similar calorific nature containing 8 per cent of protein from the *entire* barley grain. The addition of vitamine in the form of yeast did not improve the results. It is not unlikely that the milling process has removed fractions of the barley protein, residing in the outer layers of the grain, which supplement the less effective proteins of the barley endosperm. In a similar way it is known that the total proteins of wheat are superior to the proteins of the endosperm of that cereal.

Oats.

In the present series of experiments to ascertain the nutritive value of the oat proteins finely ground, commercial oat groats[7] were employed in preparing the foods. The various mixtures had the following composition.

	10 per cent protein food.	8 per cent protein food.	5 per cent protein food.
	per cent	*per cent*	*per cent*
Oat kernels	70	57	35.5
Salt mixture*	3	3	3.0
Butter fat	5	5	5.0
Starch	22	35	56.5

* The composition of the salt mixture employed is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

The results of the feeding experiments with these mixtures, as exhibited in the gains of body weight of growing rats, are shown in Charts V, VI, and VII.

The successful growth of several of the animals to large size must, we believe, be interpreted to indicate that the total protein of the oat kernel can furnish all the essential nitrogenous units if the intake of food and its concentration of protein are adequate. Very low concentrations of protein in a diet necessitate such large intakes of food to provide enough protein for growth at a normal rate that the energy intake would be inordinately large. Rats will not consume such excesses of food calories; hence, when they satisfy their energy requirement by ingestion of a food relatively poor in protein, growth is slowed in proportion to the quantity (and quality) of the *protein* actually consumed. This is evidently the limiting factor in the experiments with rations containing only 5 per cent of oat proteins.

As a rule our rats for some reason did not eat the oat foods so readily as the barley rations. The food intakes recorded in Tables I and III indicate this. Herein may lie the explanation of the failure of a considerable number to grow well. Some of

[7] The oat groats were the entire kernels of the oat seed from which the husks or glumes were removed. This material was kindly supplied to us by Mr. Cutting of the Quaker Oats Company.

TABLE III.

Oat.

Protein in food.	Rat.	Initial weight.	4 week period.			10 week period.		
			Gain in weight.	Total food intake.	Gain per gm. protein eaten.	Gain in weight.	Total food intake.	Gain per gm. protein eaten.
per cent		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
10	5700♂	65	38	248	1.5	80	616	1.3
10	5688♂	66	26	215	1.2	83	590	1.4
10	5677♂	71	21	202	1.0			
10	5693♂	70	21	209	1.0			
8	5428♂	70	48	306	2.0	115	824	1.7
8	5433♂	58	45	313*	1.8	77	702*	1.4
8	5442♂	66	30	246	1.5			
8	5461♂	66	22	204	1.3	40	501	1.0
8	5501♂	65	21	204	1.3			
8	5486♂	58	20	189	1.3	27	438	0.8
8	5464♂	69	19	235	1.0	48	638*	0.9
8	5500♂	61	19	184	1.3	24	447	0.7
8	5424♂	63	17	211	1.0	31	484	0.8
5	5484♂	67	36	338	2.1	74	839	1.8
5	5443♂	66	29	262	2.2	54	656	1.7
5	5444♂	71	27	249	2.2	59	616	1.9
5	5485♂	62	24	261	1.8	68	746	1.8
5	5490♂	63	22	250	1.8	36	608	1.2
5	5438♂	65	21	252	1.7	38	589	1.3
5	5511♂	66	21	246	1.7			
5	5429♂	80	16	294	1.1	60	746	1.6
			Oat + Vitamine.					
5	6206♂	69	15	238	1.3			
5	6213♂	70	27	226	2.4			
5	6234♂	69	25	279	1.8			

* The records for the food intakes in these experiments were unsatisfactory.

our experiments (Rats 5442, 5433, 5461, 5424, 5486, 5500, 5438, and 5490) were terminated without waiting to observe a possible subsequent improvement, because we were primarily concerned at the time with the comparison of the protein efficiency of different cereals under conditions of comparable energy intake.

Our long continued and successful results make the hypothesis of a toxic factor improbable.

It was possible that the content of water-soluble vitamine might be the limiting factor in those cases where the food intake was not large. Experiments by McCollum and his collaborators[8] indicate that 60 per cent of rolled oats in a mixture similar in energy value to our ration can supply enough of this vitamine to permit good growth. We have, nevertheless, repeated the experiments during a period of 4 weeks by furnishing a ration low in oat protein but unquestionably adequate in water-soluble vitamine.

These trials were comparable to those already described with barley (page 280), the same supply of protein-free yeast vitamine being employed. Chart XIII, as well as the statistical data (Table III), fails to indicate any advantage in the larger intake of water-soluble vitamine, so that the results with the 5 per cent oat protein food must be ascribed to the comparatively small content of protein in the diet.

McCollum[9] and his associates have recorded numerous observations upon the dietary value of the oat kernel. He states:

> "We have not yet been able to supplement oats with purified food ingredients and attain optimum results, when the oat kernel constituted from 70 to 80 per cent of the food mixture. Gelatin combined with oat proteins forms a much better protein mixture than do casein and oat proteins. . . . . We have not yet determined the cause, but it is evident that a high intake of oats over a long period causes injury to the rat. This is true also for the cow, and I believe also for swine."[10]

Our experience with respect to the value of casein and gelatin respectively has been recorded elsewhere.[1]

Why we have obtained very considerable growth upon oat diets containing 5 per cent of protein whereas McCollum and

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TABLE III.

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Simmonds[11] rarely secured maintenance upon foods of essentially similar energy content containing either 4.5 or 6.75 per cent of oat proteins (see their Chart 7) is not clear. The only conspicuous difference in the rations fed lies in the use of starch in our diets instead of dextrin and agar in the food mixtures used by McCollum and Simmonds. Their experiments in which large supplements of casein or other proteins were used have less significance in estimating the comparative value of the different cereal proteins; the quantities of the supplementary protein employed have presumably in themselves been sufficient to promote growth independently of the cereal protein used. The confusion now existing in the literature of nutrition regarding the rôle of the oat proteins *per se* (aside from any possible detrimental factor in the kernel as a whole) may be attributed to the conflicting statements published. Thus we read in one place, "The oat kernel seems to contain proteins of a poorer quality than either the maize or wheat kernel."[12] Again we read,

> "The protein of the oat kernel has a slightly higher value for growth than has that of either wheat or corn, but the amount furnished by 90 per cent of rolled oats is below the optimum for the support of growth in a rapidly growing species."[13]

Or again we are told that "the oat proteins are distinctly better than those of wheat, maize, or rice."[11]

In a recent report from the Agricultural Experiment Station of the University of Wisconsin, referring to the effect of organic nutrients on animal growth and reproduction, it is stated:

> "In every case where the oat plant was fed miserably poor offspring has resulted; for instance, with oatmeal and oat straw and butterfat; oatmeal, casein, butterfat and oat straw. This would seem to indicate that the trouble is not due to the poor protein or low vitamine content, but is a matter of actual deficiency in the mineral elements in the ration. Where the ration has been fortified with either calcium acetate or wood ashes, normal offspring has resulted. Further experiments are necessary to con-

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[11] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxii, 362.

[12] McCollum, E. V., Simmonds N., and Pitz, W., *J. Biol. Chem.*, 1917, xxix, 342.

[13] McCollum, E. V., The newer knowledge of nutrition, New York, 1919, 163.

firm this, but it is probably true that the main mineral constituent which is lacking is calcium. . . . . Oats fed with corn stover and other roughages carrying a high calcium content gave no trouble whatever."[14]

We shall, therefore, refer later to the indications offered by our own strictly comparable tests of the comparative growth-promoting value of different cereals.

Rye.

For the experiments to ascertain the nutritive value of the rye proteins the finely ground entire kernels were employed in food mixtures of the following composition.

	10 per cent protein food.	8 per cent protein food.	5 per cent protein food.
	per cent	*per cent*	*per cent*
Whole rye	80.0–85.4	64.0–69.5	40.0–42.7
Salt mixture*	3.0	3.0	3.0
Butter fat	5.0	5.0	5.0
Starch	12.0–6.6	22.5–28.0	49.3–52.0

* The composition of the salt mixture employed is given in Osborne, T. B., and Mendel, L. B., *J. Biol.Chem.*, 1919, xxxvii, 572.

The results of the feeding experiments, exhibited in gains of body weight of growing rats on these mixtures, are shown in Charts VIII, IX, and X, and Table IV.

Out of all these trials with male rats, only three, Nos. 5447, 5446, 5465, have reached a body weight of 200 gm. Many of the rats, notably those on the foods containing the higher percentages of rye, have grown well for a considerable period, during which their food intakes, as recorded later in this paper, were reasonably large and, as will be subsequently shown, the efficiency of the proteins for growth was comparable with that of the other cereal proteins included in the present study. Had our investigations been confined to a few weeks or months we probably should have failed to realize the exceptionally large mortality which subsequently involved our rye-fed animals. It is unlikely that these untoward results are due to some intercurrent disease prevalent

[14] Russell, H. L., and Morrison, F. B., *Univ. Wisconsin Agric. Exp. Station, Bull. 302*, 1919, 55.

TABLE IV.

Rye.

Protein in food.	Rat.	Initial weight.	4 week period.			10 week period.		
			Gain in weight.	Total food intake.	Gain per gm. protein eaten.	Gain in weight.	Total food intake.	Gain per gm. protein eaten.
per cent		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
10	5733♂	67	48	308	1.6	105	739	1.4
10	5737♂	71	33	275	1.2	84	723	1.2
10	5732♂	70	30	235	1.3	84	651	1.3
10	5731♂	71	30	288	1.0	69	686	1.0
8	5447♂	82	44	328	1.7	102	884	1.4
8	5522♂	67	43	295	1.8	73	688	1.3
8	5524♂	74	42	291	1.8	76	724	1.3
8	5448♂	69	37	304	1.5	75	728	1.3
8	5487♂	82	36	320	1.4	60	699	1.1
8	5523♂	75	34	304	1.4	68	709	1.2
8	5449♂	76	27	277	1.2	81	711	1.4
8	5446♂	84	26	287	1.1	77	734	1.3
5	5553♂	71	34	344	2.0	57	882*	1.3
5	5552♂	74	20	339	1.2			
5	5462♂	75	19	320	1.2	55	763	1.4
5	5465♂	73	19	293	1.3	51	737	1.4
5	5557♂	66	19	288	1.3	44	830	1.1
5	5556♂	69	18	249	1.4	39	718	1.1
5	5471♂	71	17	245	1.4	42	600	1.4
5	5472♂	69	17	274	1.2	44	669	1.3
			Rye + Vitamine.					
5	6223♂	70	21	283	1.5			
5	6226♂	69	17	278	1.2			
5	6224♂	70	15	274	1.1			

*The records for the food intakes in these experiments were unsatisfactory.

in our colony, because in that event a comparable number of deaths might have been expected among the other groups of cereal-fed animals simultaneously being investigated. The seemingly good nutrition of the rye-fed animals in the earlier periods of their life makes it unlikely that the proteins *per se* are chargeable with the untoward results subsequently exhibited by this

group of animals as a whole. What other deleterious factor, if any, may be present we are as yet unable to state.

It is not likely that the poor growth secured on the 5 per cent rye protein mixtures was due to a lack of water-soluble vitamine, inasmuch as the special series of experiments wherein 18.6 mg. of protein-free vitamine preparation from yeast were supplied daily yielded no better results (Chart XIII). The low protein content of the ration was evidently the limiting factor in growth.

The only comparable experiments which we have discovered in the literature are by McCollum, Simmonds, and Parsons[5] who state:

". . . . such cereal grains as maize, rye, and barley contain proteins of such values that when fed at 9 per cent of the food mixture, supplemented with respect to certain salts and fat-soluble A, young rats are able to grow at approximately half the normal rate."

In the composite growth chart of male animals, presented by these authors, a body weight of 200 gm. was attained. In further experiments in which rye, to the extent of 50 per cent (equivalent to 6 per cent of protein from rye) of the ration, was supplemented by flaxseed oil meal or millet seed as a further source of protein, females grew fairly well and produced young.[15] The diet was regarded as not quite satisfactory because of "the poor quality of its proteins, and shortage of fat-soluble A." Nevertheless these animals received in the diet 2 per cent of butter fat, a quantity which McCollum reports elsewhere[16] "to be sufficient for the maintenance of good growth when all other dietary factors are of good quality." The only further reference to the rye proteins which we have noted is the statement of McCollum, Simmonds, and Parsons[17] according to which

". . . . it appears that rye and flaxseed proteins in this proportion [rye proteins 6 per cent, flaxseed proteins 3 per cent] are nearly if not quite equal in value for growth to the proteins of milk."

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[15] McCollum, E. V., The newer knowledge of nutrition, New York, 1919, 175.

[16] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 162.

[17] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 160.

Wheat.

The wheat mixtures used in these experiments were prepared from the whole wheat kernel ground in the laboratory. They had the following composition.

	10 per cent protein food.	8 per cent protein food.	5 per cent protein food.
	per cent	*per cent*	*per cent*
Whole wheat	92	70	51
Salt mixture*	3	3	3
Butter fat	5	5	5
Starch	0	22	41

* The composition of the salt mixture employed is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

That growth to large adult size can be completed at a normal rate on the food containing 92 per cent of wheat has already been demonstrated by us.[18] Several litters of young were secured from females on this diet. The growth of rats kept on the wheat food of lower protein content is shown in Charts XI and XII. Several of the experiments with 8 per cent wheat protein were terminated after a few weeks of observation without reference to the final outcome. The growth of the remainder, though fairly vigorous at first, soon slowed.

The young rats of the second generation on the 10 per cent wheat protein food[19] failed to grow with normal vigor. Thus of one litter from Rat 4681 ♀, three young, 5075 ♂, 5076 ♂, 5077 ♂, died at the ages of 6, 8, and 12 months respectively without having attained a maximum weight of more than 75, 75, and 62 gm. respectively. All the young of a second litter died at an early age. Out of a litter from Rat 4577 ♀, two died very young and 5325 ♀, 5326 ♀, 5327 ♀, 5328 ♀, 5330 ♂, and 5331 ♂ survived for some time, having reached a maximum body weight of 107, 45, 57, 32, 40, and 48 gm., at 270, 88, 117, 102, 151, and 137 days

[18] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557, Chart II.

[19] The curves of growth of Rats 4577 and 4681, which bore these young, have been published. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919 xxxvii, 557, Chart II.

TABLE V.

Wheat.

Protein in food.	Rat.	Initial weight.	4 week period.			10 week period.		
			Gain in weight.	Total food intake.	Gain per gm. protein eaten.	Gain in weight.	Total food intake.	Gain per gm. protein eaten.
per cent		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
10	4669♂	60	45	322	1.4	114	810	1.4
10	4689♂	63	44	322	1.4	102	823	1.2
10	4680♂	68	39	287	1.4	92	784	1.2
8	5463♂	66	40	345	1.4	84	873	1.2
8	5469♂	65	35	346	1.3	77	873*	1.1
8	5381♂	62	34	332	1.3	83	848	1.2
8	5470♂	67	33	323	1.3	78	815	1.2
8	5382♂	67	28	274	1.3			
8	5457♂	62	23	234	1.2	46	596	1.0
8	5375♂	61	20	275	0.9	52	753	0.9
5	5711♂	76	18	289	1.2	41	714	1.1
5	5694♂	66	14	262	1.1	31	600	1.0
5	5676♂	71	9	254	0.7	29	606	1.0
5	5690♂	69	7	237	0.6	29	547	1.1
5	5416♂	61	4	207	0.4			
5	5415♂	65	4	211	0.4			
5	5399♂	76	4	271	0.3			
5	5398♂	71	4	242	0.3			
Wheat + Vitamine.								
5	6227♂	69	25	264	1.9			
5	6229♂	70	22	317	1.4			
5	6222♂	70	7	242	0.6			

* The records for the food intakes in these experiments were unsatisfactory.

respectively. Whether this outcome was due to a protein deficiency—which seems less likely—or to some other factor cannot be decided with certainty at present. McCollum, Simmonds, and Pitz[20] also state that they have not been able to make up a ration containing wheat proteins only which was adequate for

[20] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916–17, xxviii, 211.

rearing of the young. They add: "Over a wide range of protein content growth approximated the normal, but pronounced injurious effects of the ration were revealed in the reproduction records only." Some of the young (see their Chart 9) appeared normal except for their diminutive size. We have had a similar experience.[21] Incidentally we may note, however, that we have successfully raised a third generation of fertile rats on a diet in which the protein was derived solely from commercial wheat embryo.[22]

Whether the slow growth of the rats on diets containing only 5 per cent of wheat protein was due to a lack of water-soluble vitamine in the 51 per cent of wheat contained in the food mixtures can be judged by comparison with the further results obtained by furnishing a ration equally low in wheat protein, but unquestionably adequate in water-soluble vitamine. This was derived from additions of 18.6 mg. per day of protein-free vitamine preparation from yeast and employed as in the experiments with 5 per cent barley proteins (page 280). If one may judge from the food intakes and increments of body weight (Table V and Chart XIII)—which were essentially of the same order as those in the other 5 per cent wheat protein food series—the limiting factor in the wheat protein trials is to be found in the low protein content of the ration.

The Comparative Nutrient Efficiency of the Entire Proteins of the Barley, Oat, Rye, and Wheat Kernels.

The growth of our rats, on diets essentially comparable except in respect to content and source of the cereal *proteins* contained therein, show the possibilities of nutrition when any one of four commonly used cereal grains furnished the protein. To formulate a tenable comparison of the relative nutritive value of these proteins in growth it is necessary, as we have frequently pointed out before, to know the food intake. The criteria for such experiments have already been discussed elsewhere by us.[3]

[21] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 596.

[22] The food mixtures and history of the parent animals are described in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557, Chart XIV.

The tabular summaries give the essential facts in the case of those animals for which the data are reasonably accurate. In many trials, particularly with the wheat mixtures, so many animals spilled food as to render the statistics for food intake incomplete; hence they have been rejected in every case. The figures recorded are the selected residual complete data from a large number of trials. The growth results have been calculated from animals beginning at a body weight of about 70 to 80 gm. Calculations have been made to include the subsequent first 4 weeks; and likewise the first 10 weeks, during which a rat normally more than doubles its body weight. The composition of the foods from the standpoint of calorie value was substantially the same, starch being substituted for cereal whenever the content of the latter was reduced in the ration; hence the energy intake in the experiments with foods containing different protein concentrations must have been practically proportional to the total intake of food. The graphic records of the body weights of all these animals will be found in Charts I to XIII.

If the figures representing gains per gm. of protein eaten are contrasted for the first periods of 4 weeks a slight advantage might seem to accrue to the barley proteins. This advantage is scarcely apparent, however, when the longer period (10 weeks) is taken into account. Obviously the number of trials is far too small to permit the use of such limited statistics for computation of averages. Until a far greater refinement of the method is secured it would appear, on the whole, that the proteins of the four cereals studied are not widely different in their efficiency in promoting growth. In contrasting the different groups of animals from a standpoint of their subsequent history, however, it seems as if the barley-fed animals have, if anything, grown best.

The comparative equality of the four types of cereal proteins just recorded becomes the more striking when the gains which they promote per gm. of protein consumed are contrasted with those secured within comparable periods of growth by the use of proteins from other sources. Thus, in feeding rats of similar initial weight for periods of 8 weeks, gains per gm. of protein eaten amounted to more than 2.3 gm. for lactalbumin and 1.7 gm. for casein. The advantage of the addition of more efficient supplementary protein to the cereal protein has been discussed elsewhere.[2]

In somewhat similar experiments on the comparative nutritive value, in growth, of proteins of wheat endosperm (flour and wheat gluten) with and without supplements of animal protein (meat, milk, egg), the best gains per gm. of protein eaten were not much larger, during a 4 week period, than those here reported for the four cereals. The outcome with the wheat endosperm proteins alone was decidedly poorer, as the following summary shows.

Summary.*

	Per cent of protein in food.	Gain of body weight per gm. of protein.
		gm.
Flour + egg	14.8	2.00
	10.3	1.80
" + milk	14.8	1.67
	10.3	1.73
" + meat	14.8	1.73
	10.3	1.47
" + " + yeast	10.3	1.66
" + gluten	14.8	0.50

* Quoted from Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 584.

We were surprised to find that the *efficiency* of the *entire* wheat kernel, as well as that of the other cereal grains studied, without supplementary proteins, was so far superior to that ascertained by us earlier in the study of the *endosperm*. The utilization data secured by us testify to the unexpected availability for growth of the proteins of these whole cereals. This is in harmony with the recent findings of Sherman and his collaborators[23] in the study of the maintenance metabolism of adults as shown by nitrogen balance experiments on diets in which the proteins of oats and maize contributed a large portion of the protein intake. They assert:

[23] Sherman, H. C., Wheeler, L., and Yates, A. B., *J. Biol. Chem.*, 1918, xxxiv, 383. Sherman, H. C., and Winters, J. C., *J. Biol. Chem.*, 1918, xxxv, 301. Sherman, H. C., Winters, J. C., and Phillips, V., *J. Biol. Chem.*, 1919, xxxix, 53.

" . . . . the proteins of oats and maize are of virtually equal nutritive efficiency; and this is true whether the proteins in question constitute practically the sole nitrogenous food or are supplemented by a constant small amount of milk protein. That nitrogen equilibrium was maintained with such low protein intake in the latter experiments, and was so closely approached in the former, shows that the proteins of both maize and oatmeal were very efficiently utilized in the maintenance metabolism of these healthy adults. . . . . For the purposes of practical dieteties equal weights of oat and maize proteins may be regarded as essentially equal in value, and even the minimum amount of milk which can possibly be regarded as permissible in the light of our present knowledge of nutrition, will apparently so supplement the proteins of either the maize or oat kernel as to make them function with an efficiency comparable with that of the average protein of mixed diet in the maintenance metabolism of man."

The ideal of equivalent calorie intakes during comparable periods of time in the growth experiments to compare the efficiency of proteins from different sources has by no means been realized in the trials which we have recorded in this paper. At best our experiments show the possibility of normal growth for long periods where no other proteins than those furnished from these cereals are available. They also indicate a surprisingly good efficiency in the most favorable experiments; but inasmuch as many ultimate failures of growth and well being, and numerous deaths, were encountered in the later periods of many of the experiments with some of these cereals it will be necessary to take into account such unknown factors as may have been responsible for these ultimately unfavorable results. We have observed sufficient, successful, prolonged growth in the case of all the cereal studies to make it unlikely, not to say improbable, that the protein factor is responsible for this untoward outcome of many of the cereal experiments.

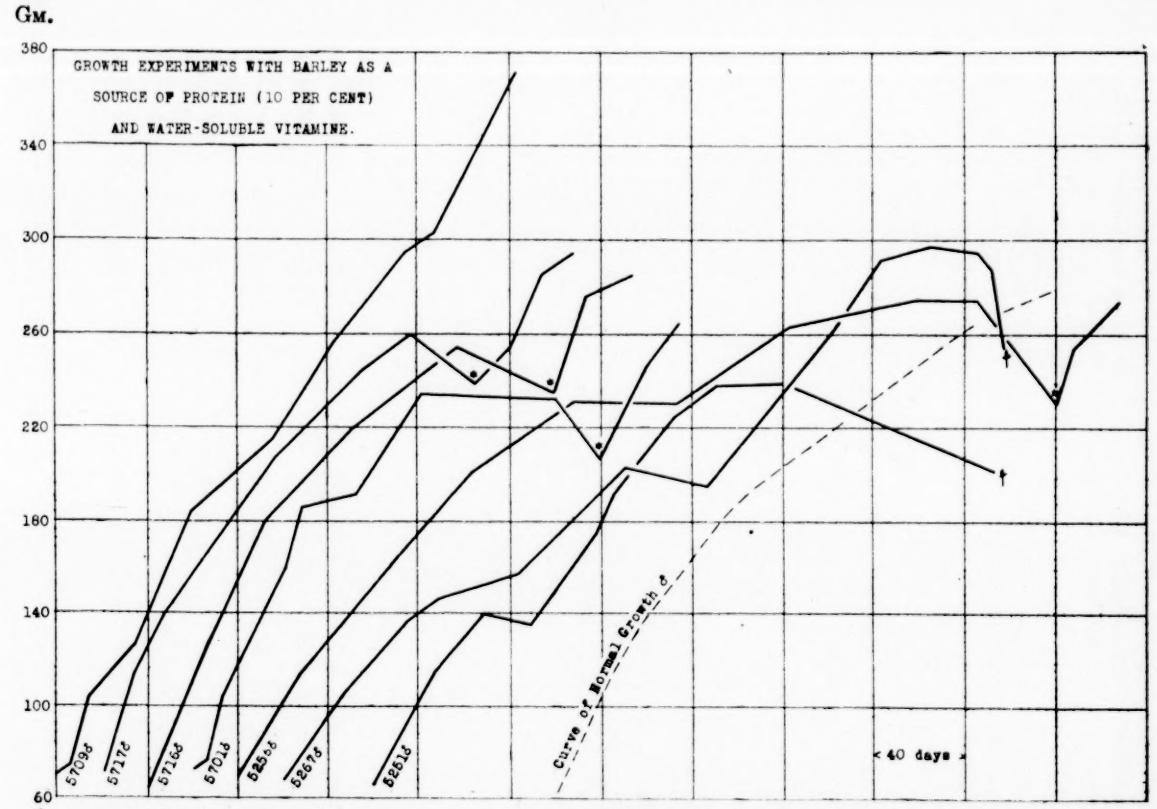


CHART I. Showing the growth of rats on an otherwise adequate diet in which all the protein (10 per cent) and the water-soluble vitamine were furnished by barley, which formed 92 per cent of the food mixture. The asterisks (*) indicate the beginning of the period when the chaff was mostly removed by sifting the barley during grinding, whereupon the animals appeared to eat the food more greedily, with resulting increments in body weight.

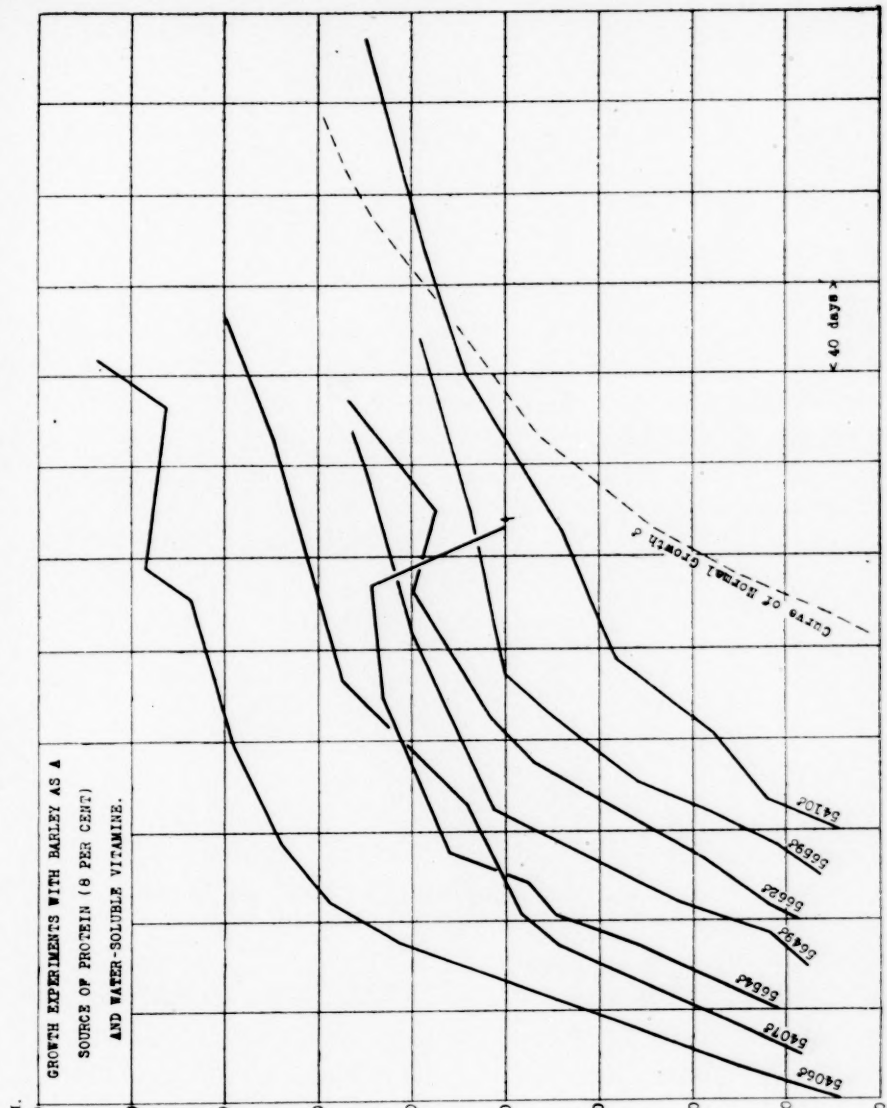


CHART II. Showing the growth of rats on an otherwise adequate diet in which all the protein (8 per cent) and the water-soluble vitamine were furnished by barley, which formed 72 to 75.5 per cent of the food mixture.

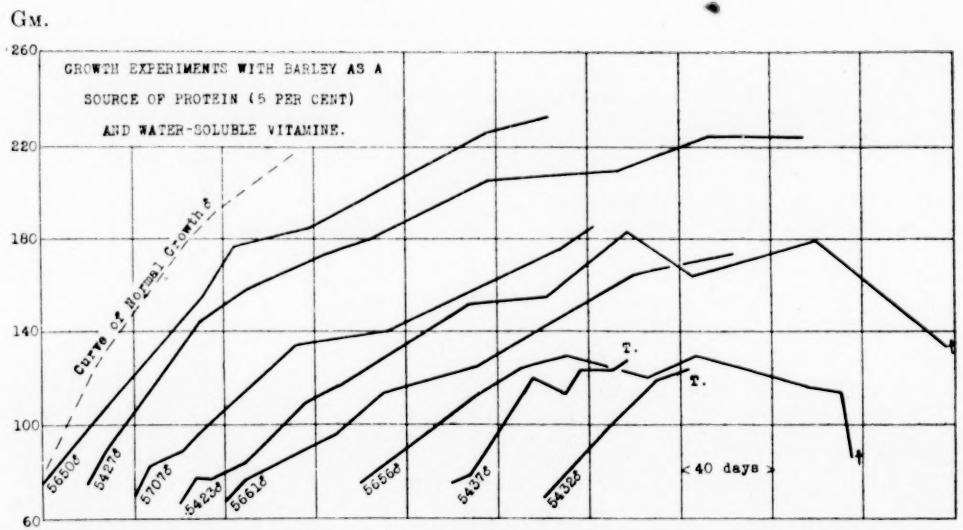


CHART III. Showing the growth of rats on an otherwise adequate diet in which all the protein (5 per cent) and the water-soluble vitamine were furnished by barley, which formed 45 to 47 per cent of the food mixture. Some of the experiments were terminated (T.) early without reference to the final outcome of the tests.

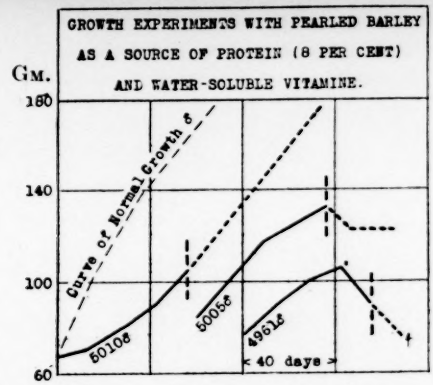


Chart IV. Showing the inferior growth of animals on an otherwise adequate diet in which all the protein (8 per cent) and the water-soluble vitamine were furnished by pearled barley, which formed 92 per cent of the food mixture. These results should be contrasted with the curves in Chart II. The failure to grow more vigorously was apparently not due to a lack of water-soluble vitamine in the preparation, since the addition of 0.2 gm. of dried brewery yeast during the period indicated by the interrupted line failed to promote any noteworthy increase in the rate of growth. This quantity of yeast has been demonstrated in repeated comparable experiments to furnish sufficient water-soluble vitamine for growth at a normal rate.

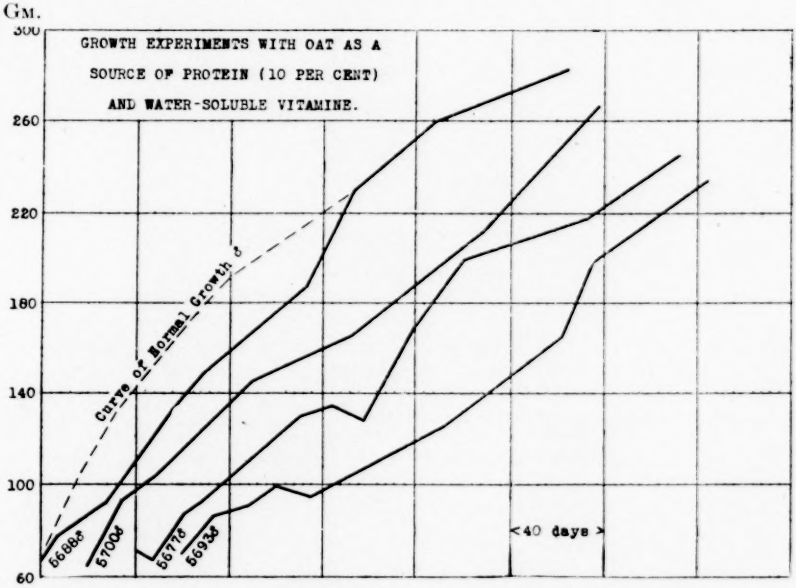
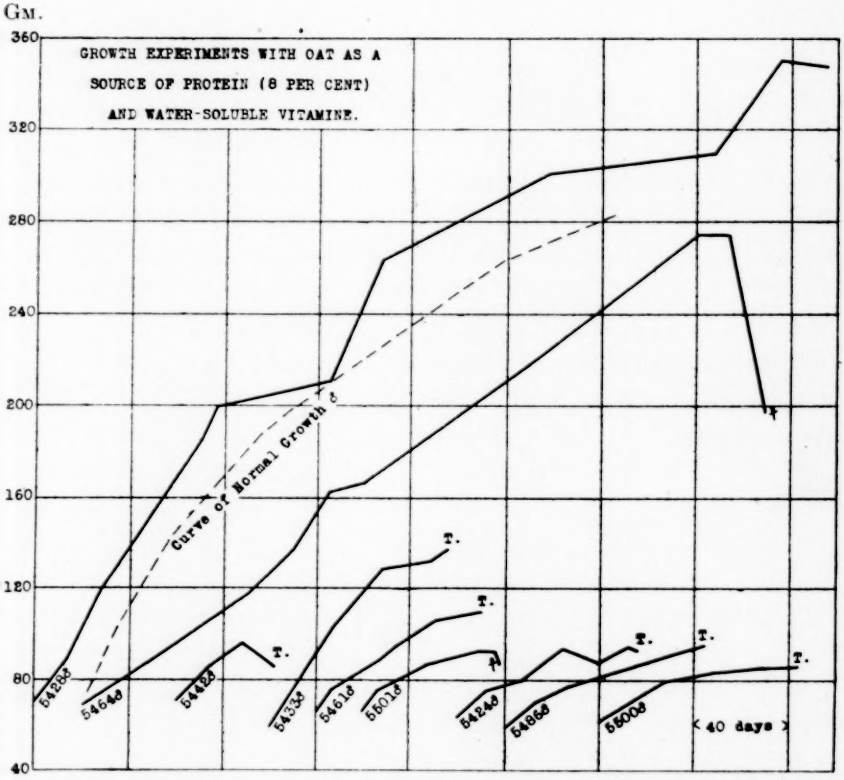


Chart V. Showing the growth of rats on an otherwise adequate diet in which all the protein (10 per cent) and the water-soluble vitamine were furnished by oat kernel, which formed 70 per cent of the food mixture.


GM.
360
320
280
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GROWTH EXPERIMENTS WITH OAT AS A
SOURCE OF PROTEIN (8 PER CENT)
AND WATER-SOLUBLE VITAMINE.
Curve of Normal Growth
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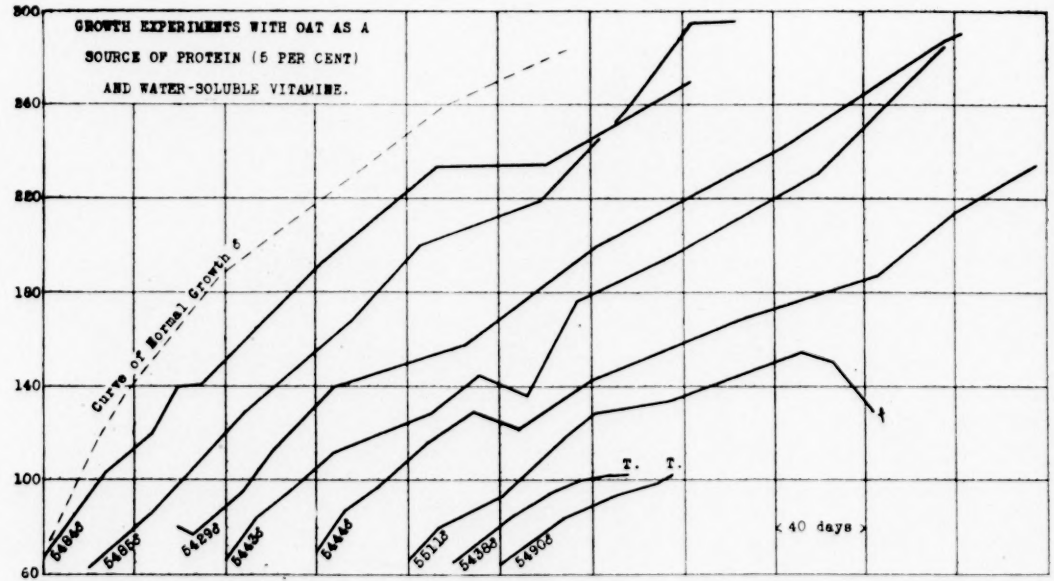

CHART VII. Showing the growth of rats on an otherwise adequate diet in which all the protein (5 per cent) and the water-soluble vitamine were furnished by the oat kernel, which formed 35.5 per cent of the food mixture. Some of the experiments were terminated (T.) early without reference to the final outcome of the tests.

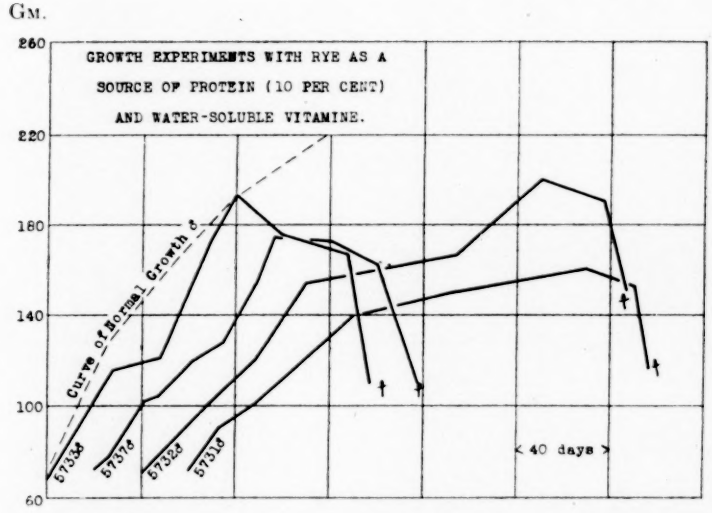

CHART VIII. Showing the growth of rats on an otherwise adequate diet in which all the protein (10 per cent) and the water-soluble vitamine were furnished by whole rye, which formed 80 to 85.4 per cent of the food mixture. The high mortality in the experiment is referred to in the text.

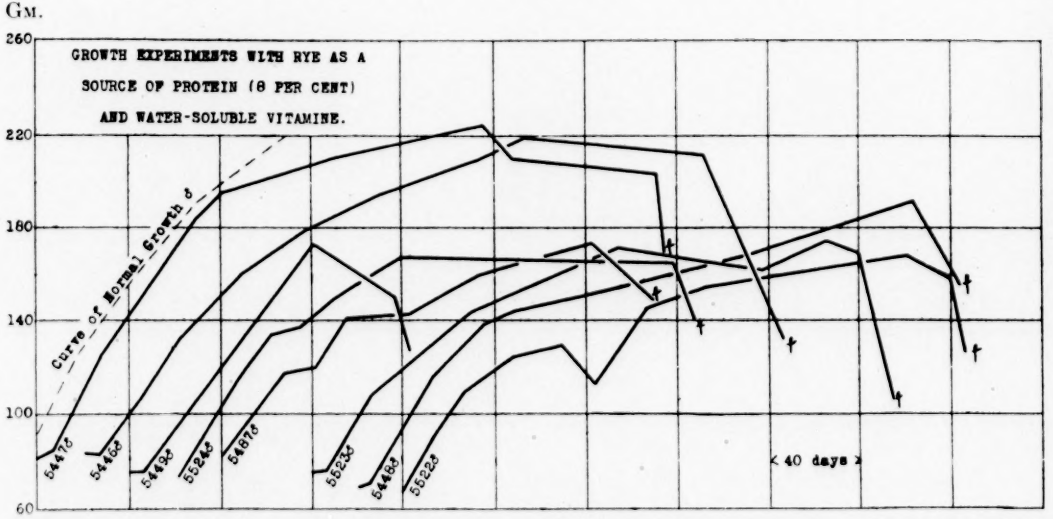

CHART IX. Showing the growth of rats on an otherwise adequate diet in which all the protein (8 per cent) and the water-soluble vitamine were furnished by whole rye, which formed 64 to 69.5 per cent of the food mixture. The high mortality in the experiment is referred to in the text.

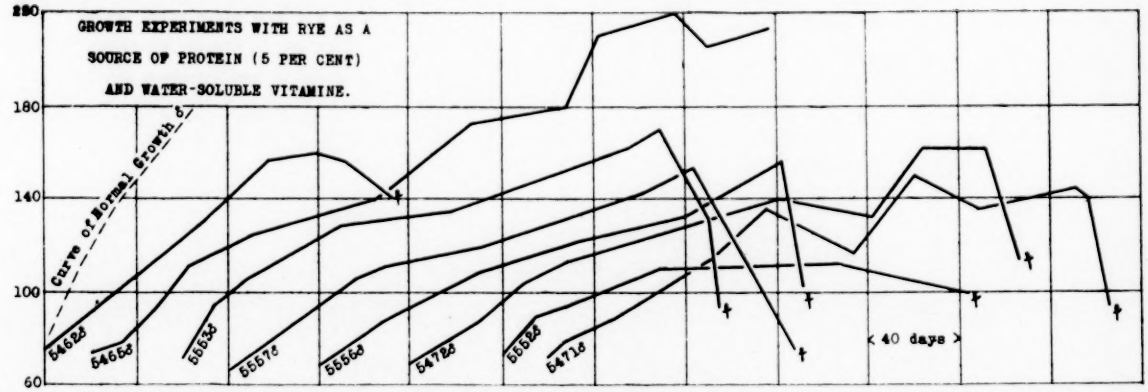

CHART X. Showing the growth of rats on an otherwise adequate diet in which all the protein (5 per cent) and the water-soluble vitamine were furnished by whole rye, which formed 40 to 42.7 per cent of the food mixture. The high mortality in the experiment is referred to in the text.

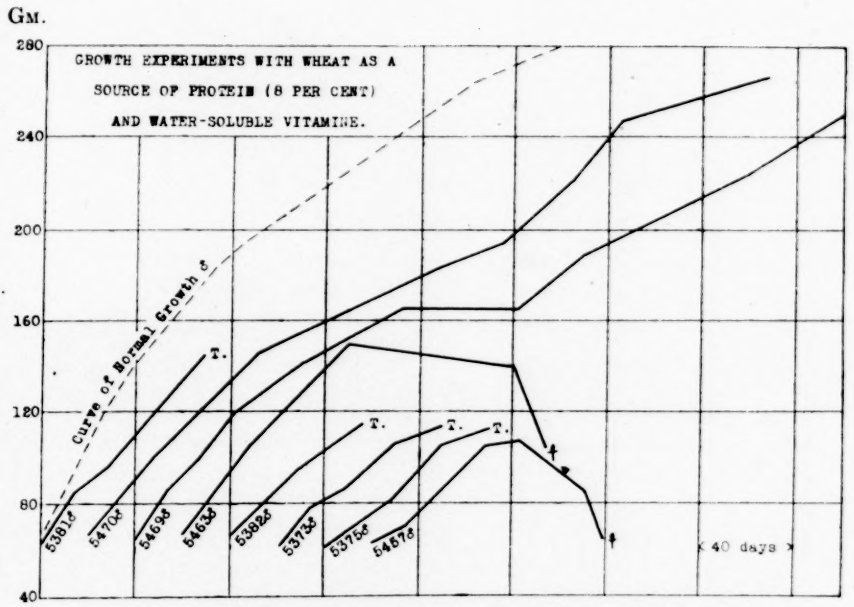

CHART XI. Showing the growth of rats on an otherwise adequate diet in which all the protein (8 per cent) and the water-soluble vitamine were furnished by whole wheat, which formed 70 per cent of the food mixture. Some of the experiments were terminated (T.) early without reference to the final outcome of the tests. The normal growth and reproduction of rats on a diet in which the protein and water-soluble vitamine are furnished by whole wheat are indicated by Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557, Chart II.

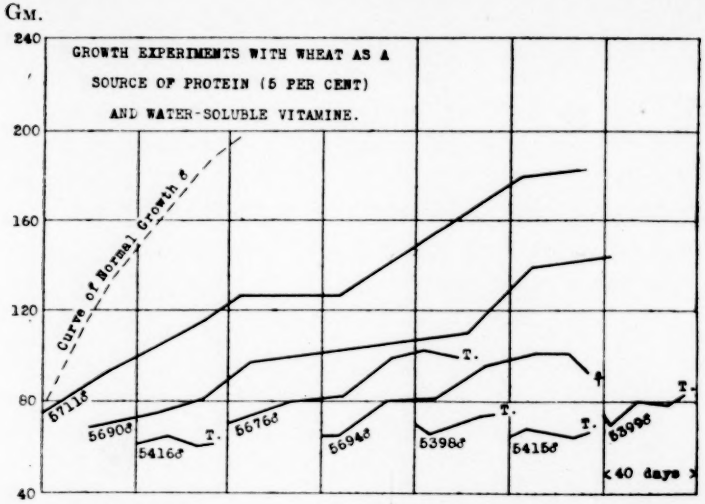

Chart XII. Showing the growth of rats on an otherwise adequate diet in which all the protein (5 per cent) and the water-soluble vitamine were furnished by whole wheat, which formed 51 per cent of the food mixture. Some of the experiments were terminated (T.) early without reference to the final outcome of the tests.

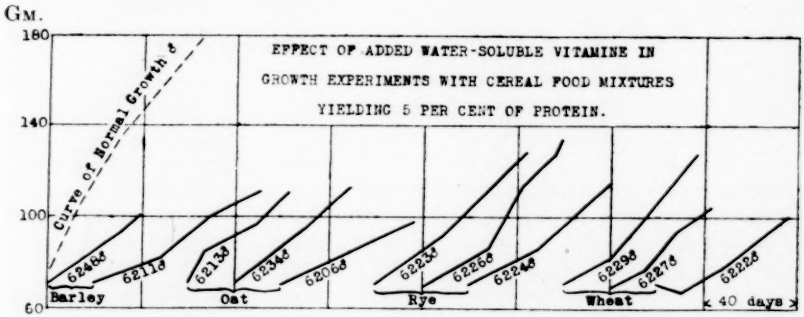

Chart XIII. Showing the effect of daily additions of a protein-free preparation of water-soluble vitamine from yeast, fed apart from the food mixture, which contained 5 per cent of protein furnished by barley, oat, rye, or wheat. The results should be contrasted with those from the comparable experiments on Charts III, VII, X, and XII respectively. They indicate that the failure to grow better on diets containing the smaller proportions of grains, equivalent to only 5 per cent of cereal protein, was not due to the lack of water-soluble vitamine in these instances. The vitamine preparation used is described by Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.

EFFECT OF THE CHLORINE SUBSTITUTION PRODUCTS OF METHANE, ACETALDEHYDE, AND OF SODIUM ACETATE ON CATALASE PRODUCTION.*

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(Received for publication, January 13, 1920.)

It is recognized that the introduction of chlorine into the molecule of an aliphatic narcotic greatly enhances its effect as a narcotic. Snow (1), Bert (2), Arloing (3), Verworn (4), and others have shown that, as a rule, oxidation is decreased during anesthesia and hence the conclusion has been drawn that narcosis may be due to the inhibition or interference with oxidation, while Crile (5) claims that it is due to the acidosis arising from diminished or defective oxidation. We (6) found that narcotics decrease catalase, an enzyme possessing the property of liberating oxygen from hydrogen peroxide, by diminishing its output from the liver and by direct destruction of the enzyme, and hence concluded that the diminished oxidation during narcosis may be due to the decrease in catalase. It was also found that glycocoll, in keeping with Lusk's observation (7) that this amino-acid increased oxidation in the body, produced a very great increase in catalase. Acetic acid or its sodium salt, a substance closely related chemically to glycocoll, was also found to produce a great increase in catalase by stimulating the alimentary glands, particularly the liver, to an increased output of this enzyme.

The present investigation was begun in an attempt to determine whether the introduction of increasing amounts of chlorine into the methane molecule would increase its destructive effect on catalase, and whether the introduction of increasing amounts

* An abstract of this paper was read at the annual meeting of the Federation of American Societies for Experimental Biology, Cincinnati, December, 1919.

of chlorine into the acetic acid molecule would diminish its effectiveness in producing an increase in catalase. The animals used were dogs, rabbits, and cats. The substances used were methane (CH_4), monochloromethane (CH_3Cl), dichloromethane (CH_2Cl_2), trichloromethane (chloroform, $CHCl_3$), tetrachloromethane (CCl_4); acetaldehyde (CH_3CHO), chloral (CCl_3CHO); sodium monochloroacetate ($CH_2ClCOONa$), sodium dichloroacetate ($CHCl_2COONa$), and sodium trichloroacetate (CCl_3COONa). The amounts of the substances will be given in the description of the experiments. The catalase was determined by adding 0.5 cc. of blood to diluted hydrogen peroxide at approximately 22°C. in a bottle, and the amount of oxygen gas liberated in 10 minutes was taken as a measure of the amount of catalase in the 0.5 cc. of blood. Account was taken in these experiments and corrections were made for the inhibiting action of acids and acid salts on catalase as observed by Jacobson (8), Loevenhart and Kastle (9), Issajew (10), Senter (11), Winternitz and Rogers (12), Mendel and Leavenworth (13), and Bodansky (14), as well as the effect of alkalies and alkaline salts.

In Fig. 1 are shown the effects of methane, acetaldehyde, and sodium acetate together with that of their chlorine substitution products on the blood catalase *in vivo* as well as *in vitro*. The figures along the abscissæ indicate time in minutes and the figures along the ordinate, percentage increase or decrease in catalase. The trichloromethane or chloroform, and tetrachloromethane were administered by bubbling air through these substances in a bottle which was connected by a rubber tube to a cone adjusted over the snout of the animal, while the methane, mono- and dichloromethane were led directly from the generator into an inverted glass vessel containing the animal. The animals used were rabbits. The catalase in 0.5 cc. of blood from the jugular vein was determined before as well as at intervals after the administration of the substances. In the chart it may be seen that methane (CH_4) had little or no effect on the blood catalase; monochloromethane (CH_3Cl) decreased it 22 per cent in 45 minutes; trichloromethane (chloroform, $CHCl_3$), 32 per cent; and tetrachloromethane (CCl_4), 37 per cent. From these figures it may be seen that the more chlorine there is in the methane molecule, the more effective it becomes in decreasing the blood catalase.

It may be seen further in the chart that glycocoll (CH_2NH_2-COOH) increased the blood catalase 52 per cent in 60 minutes; sodium acetate (CH_3COONa), 42 per cent; sodium monochloroacetate ($CH_2ClCOONa$), 37 per cent; sodium dichloroacetate ($CHCl_2$-COONa), 23 per cent; and sodium trichloroacetate (CCl_3COONa) produced practically no change in the blood catalase. Cats were used in these experiments. The amounts of the substances used were 10 gm. per kilo dissolved in 75 cc. of water. The substances were introduced into the upper part of the small intestine and the blood from the jugular vein was used for the catalase determinations. It may also be seen that 0.6 gm. per kilo of acetaldehyde (CH_3CHO) decreased the catalase 12 per cent, and that a similar amount per kilo of its chlorine substitution product, chloral (CCl_3CHO), decreased the catalase 22 per cent in 90 minutes. The animals used in these experiments were rabbits and the substances, dissolved in 50 cc. of water were introduced by means of a stomach tube.

The second part of this paper is concerned with determining the mode of action of the chlorine substitution products of methane and acetaldehyde in producing a decrease and of sodium acetate in producing an increase in catalase and also in finding an explanation for the fact that the introduction of chlorine into the acetic acid molecule diminishes its effectiveness in increasing catalase. In Fig. 1 under "*in vitro*" are shown the effects on the catalase of cat's blood when exposed to methane and its chlorine substitution products. The amount of defibrinated blood used was 5 cc., which was poured into a glass vessel in which it formed a layer about 2 mm. thick. Such preparations were exposed to methane and to its chlorine substitution products at 40°C. for the times indicated in the chart. It may be seen that the exposure of the blood to methane had practically no effect on its catalase; the exposure to monochloromethane gas decreased the catalase very little in 60 minutes; the exposure to dichloromethane decreased it 21 per cent in 15 minutes and 62 per cent in 45 minutes; the exposure to trichloromethane gas decreased it 48 per cent in 15 minutes and 76 per cent in 45 minutes. From these figures it is clear that increasing the amount of chlorine in methane increased its destructive effect on catalase *in vitro*. It may be seen further in the chart that neither sodium acetate nor any of its chlorine sub-

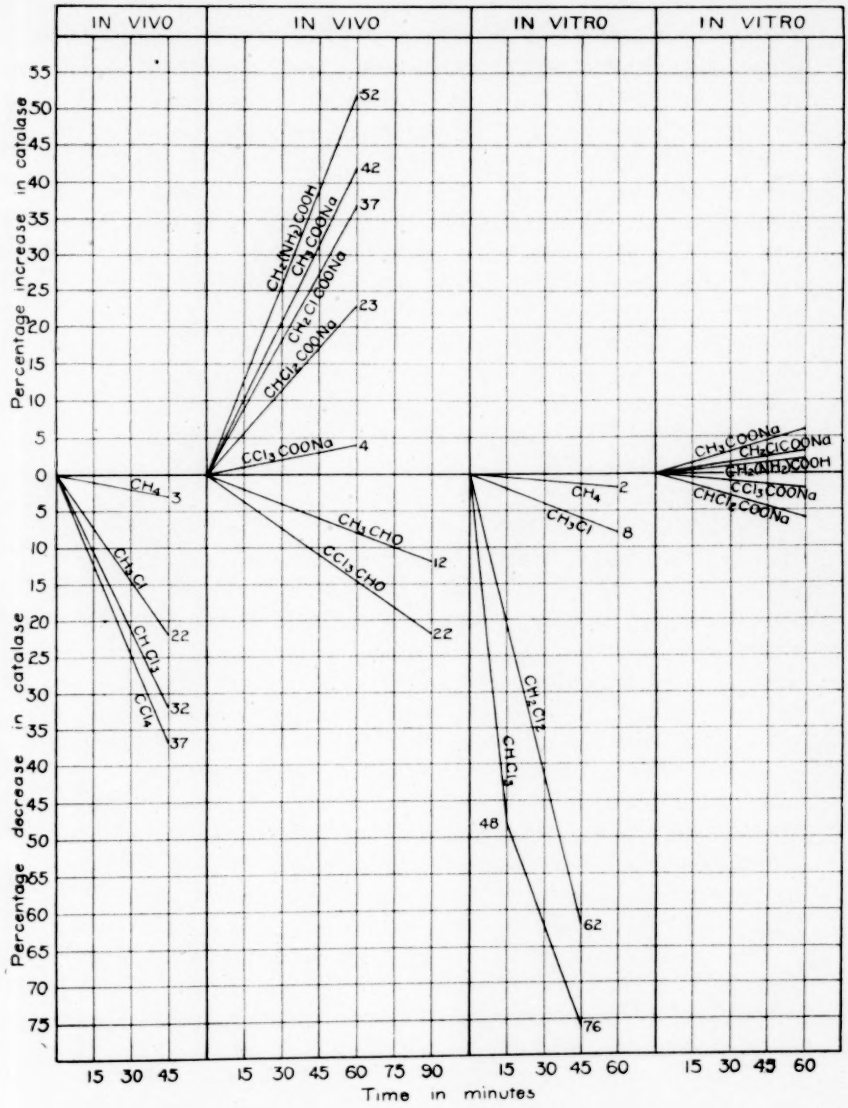

FIG. 1. Curves showing effect of the substances named in the chart on the catalase content of the blood.

stitution products had any effect on catalase *in vitro*. 100 mg. of each of these substances were added to 5 cc. of defibrinated cat's blood and the catalase determinations, using neutral hydrogen peroxide, were made at the intervals indicated in the chart.

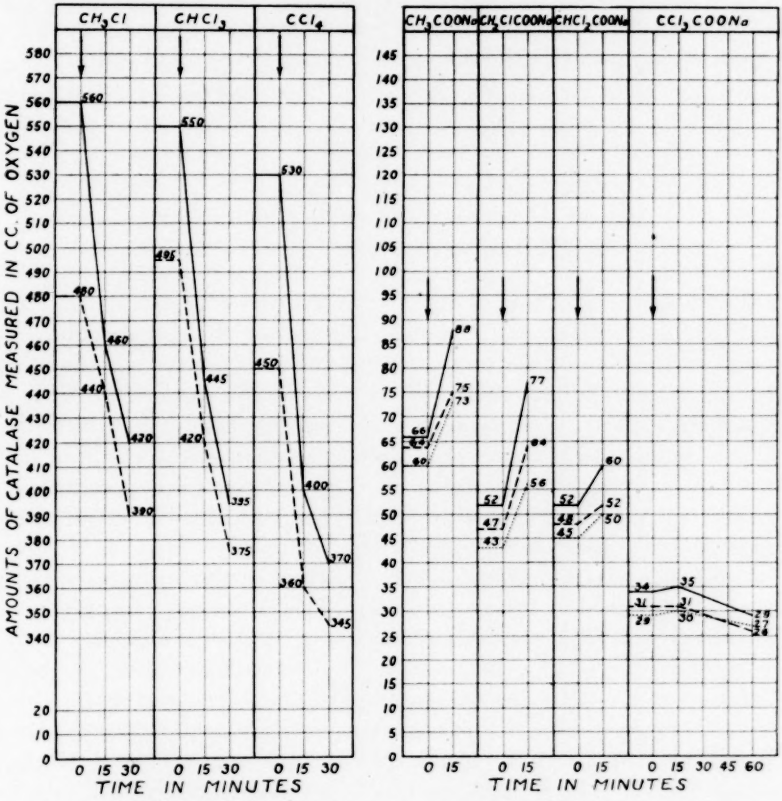

FIG. 2. Curves showing effect of the substances named in the chart on the catalase content of the blood. The continuous line curves show amount of catalase in the blood of the liver, the dash line curves the amount in the blood of the portal vein, and the dotted line curves the amount in the blood of the jugular vein.

In Fig. 2 are shown the effects of the administration of the chlorine substitution products of methane and of sodium acetate on the catalase of the blood of the liver, portal, and jugular veins.

The continuous line curves were constructed from data obtained from the blood of the liver, the dash line curves from the blood of the portal, and the dotted line curves from the blood of the jugular vein. The chloroform and tetrachloromethane were administered by bubbling air through these substances in a bottle which was connected by a rubber tube to a cone adjusted over the snout of the animal while the monochloromethane was led directly from the generator to the animal. These substances were administered in as concentrated form and in as large amounts as could be done and at the same time keep the animal alive. The animals used for the study of these chlorine substitution products were cats. It may be seen under monochloromethane (CH_3Cl) that previous to the use of this material 0.5 cc. of blood from the liver liberated 560 cc. of oxygen from hydrogen peroxide in 10 minutes and that 0.5 cc. of blood from the portal vein of the same animal liberated 480 cc.; after 15 minutes use of the monochloromethane the blood of the liver liberated 460 cc. of oxygen and that of the portal vein 440 cc. and after 30 minutes the blood of the liver liberated 420 cc. of oxygen and that of the portal vein 390 cc. Under trichloromethane ($CHCl_3$) and tetrachloromethane (CCl_4) it may be seen that these substances also produced a decrease in the catalase of the blood of the liver and of the jugular vein. By comparing these figures it is evident that the catalase content of the blood of the liver in all the animals used was much greater than that of the blood of the portal vein. This is taken to mean that the liver is putting out catalase continuously into the blood. By comparing the decreases produced by these substances in the catalase of the blood of the liver and of the portal vein it may be seen that they produced a much greater decrease in the blood of the liver than they did in the blood of the portal vein. This is taken to mean that these substances were decreasing the output of catalase from the liver. It should be mentioned in this connection that Becht (15) has repeated some of our work on the effect of narcotics on the blood catalase and claims that catalase is increased instead of being decreased during narcosis.

In Fig. 2 are shown also the effects of sodium acetate and its chlorine substitution products on the catalase of the blood of the liver, portal, and jugular veins. The animals used were dogs. After opening the abdominal wall of these animals with the use

of ether anesthesia, 10 gm. per kilo in 300 cc. of water were introduced into the upper part of the small intestine. By comparing these figures it may be seen that sodium acetate produced an increase in the catalase of the blood and that this increase was greater in the blood of the liver than it was in the blood of the portal and jugular veins. This suggests that the sodium acetate was stimulating the liver to an increased output of catalase. Similarly it may be seen that the mono- and dichlorine substitution products of sodium acetate produced an increase in catalase in 15 minutes while the trichlorine substitution product produced a small decrease in 60 minutes. By comparing these figures it may be seen that the effect of the introduction of chlorine into the sodium acetate molecule was to decrease its effectiveness in stimulating the liver to an increased output of catalase.

SUMMARY.

1. The more chlorine that is introduced into the methane molecule the more effective it becomes in decreasing catalase. Similarly, the strong narcotic chloral, a trichlorine substitution product of acetaldehyde, decreases catalase more than does acetaldehyde, a weaker narcotic. The ingestion of sodium acetate produces an increase in catalase. The introduction of increasing amounts of chlorine into the sodium acetate molecule renders it less effective in increasing catalase.

2. The chlorine substitution products of methane decrease catalase by direct destruction of the enzyme and by decreasing its output from the liver. Sodium acetate produces an increase in catalase by stimulating the liver to an increased output. The introduction of chlorine into the molecule renders it less effective as a stimulant on catalase production.

3. The increase in oxidation following the ingestion of glycocoll or a closely related compound, acetic acid (sodium salt), is attributed to the increase in catalase. The decrease in oxidation arising during chloroform and chloral anesthesia and assumed by some to be the cause of the narcosis is attributed to the decrease in catalase.

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AN IMPROVED VOLUMETRIC PUMP FOR CONTINUOUS INTRAVENOUS INJECTIONS.

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Plate 3.

(Received for publication, January 16, 1920.)

A machine described earlier[1] consisted essentially of a single glass syringe fitted with a two-way valve, syringe and valve being operated by an electric motor acting through a worm gear, eccentrics, and rods. The discharge of the pump was controlled coarsely by setting the stroke of the piston by means of a system of levers and more exactly through control of the motor speed by means of a rheostat. This system involved the use of variable speed motors and changing motor speeds, with the inherent disadvantages which this implies from the standpoint of uniform performance.

The present machine mounts two glass syringes or cylinders each fitted with a two-way valve. Both pumps are run by one motor acting as before through a worm gear, eccentrics, etc. The former method of adjusting the stroke is displaced by a new device which is applied separately to each piston rod. The new device is simpler and much more accurate than the old and permits the stroke of either piston to be set independently in a few seconds at the desired length while the machine is running or stopped. Owing to the stroke adjustment it becomes unnecessary to alter the speed of the motor during an experiment, thus making it possible to drop the variable speed motor and rheostat in favor of a motor of constant speed type with the decided advantage that the operator's attention is not required to secure uniform performances. With a half-horse-power "Synchronous" motor, long experiments involving repeated changes in the rate of dis-

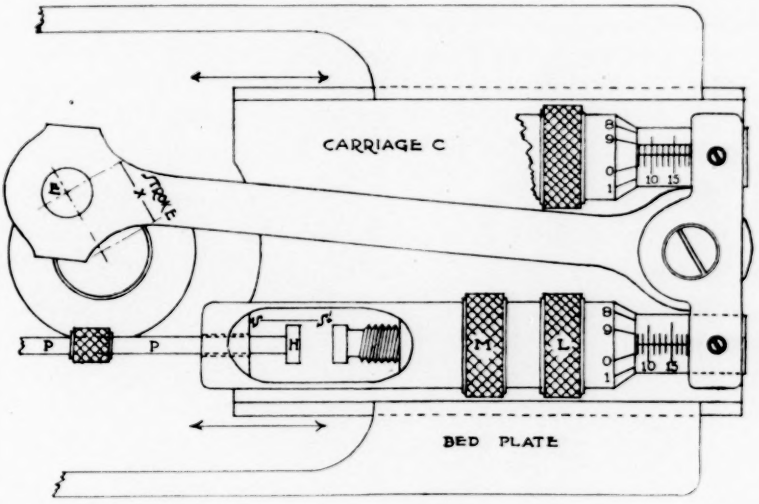
[1] Woodyatt, R. T., *J. Biol. Chem.*, 1917, xxix, 355.

charge from one or both cylinders have been conducted easily and with negligible fluctuations of the motor speed.

The stroke adjustment is illustrated in Text-fig. 1. The motion of the motor is transmitted through a worm gear to the eccentric E and by the eccentric rod to a carriage plate C. The carriage C moves in a grooved bed plate in the direction of the arrows making a stroke equal to the stroke X of the eccentric. The diagram shows the piston rod P broken off at its point of emergence from the pump cylinder. The piston rod is jointed for convenience in taking the machine apart and terminates in a hard steel head H standing free in the space SS′. This space is the measuring gap in a machinist's micrometer modified for the purpose. The micrometer is fixed by a mounting on the right to the carriage itself and projects to the left over the plane of the carriage with clearance to permit of its operation. The distance SS′ can be given any value desired by loosening the lock nut L and turning the milled collar M which causes the surface S to move toward or away from S′. When the machine is in motion it will be seen that the distance SS′ can be made so great that after one complete stroke the head H will have been pushed as far to the left as the extreme forward motion of S′ will permit, after which it will remain stationary, S′ just touching it at the end of each subsequent forward stroke while on the back stroke S will merely approach H. But as the gap SS′ is shortened by turning M so that S approaches S′ a position may be found in which at the end of the back stroke S will also just touch H. With this setting of the micrometer, H is just touched but not moved on the forward stroke by S′ and on the back stroke by S. This position corresponds to 0 on the scale and collar of the micrometer. The carriage now moves back and forth through the distance X while none of its motion is imparted to the piston although while the piston remains stationary the valves are turned. Now if S is set 1 cm. closer to S′ as read directly on the scale and collar, then H will be displaced 1 cm. on the back stroke and on the forward stroke will return to its former position, and so on for any setting within the range of the apparatus. The micrometer reads to 0.04 mm.

It will be noted that with this device the head H and so the piston rod and piston are free during the interval after the surface

S′ having pushed H to the limit is receding and while the surface S is approaching H but is not yet in contact with it. This interval follows immediately upon the completion of the systole of the pump when the pressure of the fluid in the discharge tube leading from the pump is highest. If sufficient back pressure develops it may kick back the piston before the valve cuts off the communication thus destroying the quantitative character of the pump and reducing the total discharge. There is a similar interval at the end of the diastole during which the pressure of the gravity feed



Text-Fig. 1. Improved volumetric pump for continuous intravenous injections. The actual length is 10 inches over all.

if such is used might move a loose piston and allow the cylinder to take in more than the indicated volume of fluid at each stroke. These sources of error are eliminated by a friction check on the piston rod which makes it impossible for the piston to move except in response to impacts of the surface S and S′.

The present machine has the advantages of two single machines of the earlier type in that it permits the simultaneous injection of one or two liquids into one or two discharge tubes, both constantly at different rates bearing known ratios, one constantly

at one rate with one varying, or both varying as desired. The accuracy and evenness of performance as well as the convenience of operation are much higher than in the older machine. Having a motor which runs always at the same speed, a cylinder (syringe) is calibrated by direct observation of the total volume which it discharges during periods of 15 minutes to 1 hour, with the stroke set at 0, at 15 mm., and at two or more intermediate points. In plotting the results they are found to fall on a straight line and the chart so formed indicates the setting of the micrometer necessary for the delivery of any desired volume per hour. When set to deliver a given volume in an hour, the results with the present motor fed from an ordinary power circuit have frequently fallen within 0.1 to 0.3 cc. of the desired total.

The machine was made by William Gaertner and Company, 5345 Lake Park Avenue, Chicago, Illinois, and is illustrated in Fig. 1.

EXPLANATION OF PLATE 3.

FIG. 1. Assembled machine. The syringes and valves are demountable without tools for cleansing and sterilization purposes. The large screw head at the left and below is at the end of the worm shaft bearing. The motor shaft is coupled to the opposite end of the worm shaft.

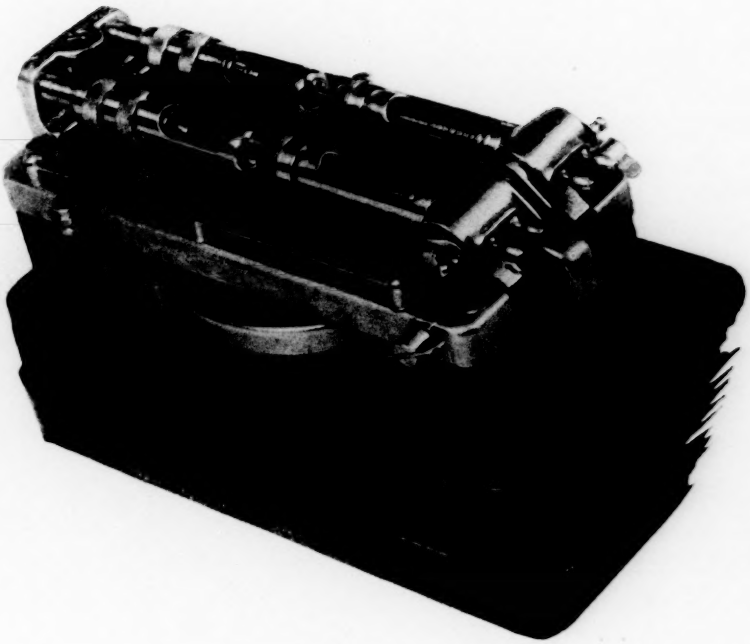


FIG. 1.

(Woodyatt: Pump for intravenous injections.)

BIOCHEMISTRY OF THE ACETONE AND BUTYL ALCOHOL FERMENTATION OF STARCH BY BACILLUS GRANULOBACTER PECTINOVORUM.

By HORACE B. SPEAKMAN.

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(Received for publication, January 10, 1920.)

During the last 4 years a considerable amount of research, chiefly along industrial lines, has been performed in connection with the production of acetone together with various alcohols by the fermentation of carbohydrate media, using cultures of different bacteria. Some of this work has already been described in the literature (1, 2). Contributions have also been made to our knowledge of the biochemistry of these important processes. Northrop and his collaborators (3) have studied in detail the behavior of *Bacillus acetoethylicum* in standard media, and also the influence of certain factors, namely the reaction of the media, the size of the inoculation, the supply of air and nitrogen (peptone), and the temperature of the medium, on the growth of the organism and the yields of acetone and ethyl alcohol. They record that the principal acid produced during the fermentation is formic. At a conference held in London during the past year the position regarding the production of acetone with butyl alcohol by fermentation methods was discussed (4). More recently Fred and his collaborators (5) have shown that during the fermentation of xylose a mixture of acetic and lactic acids is produced, using cultures of bacteria isolated from manure, silage, etc.

An effort has been made during this investigation to identify and study the rôle of the intermediate compounds formed during the fermentation of starch by *Bacillus granulobacter pectinovorum*.[1]

[1] The culture used in the research has been developed from tubes received from Dr. C. Weizman in 1915 when it was my privilege to be associated with him in the early stages of the more general investigation.

Products of the Fermentation.

At the meeting in London the percentage yields of acetone and butyl alcohol were given as 7 and 14 per cent respectively of the weight of maize meal used. During the last 3 years very large volumes of these two compounds were prepared by this process in Toronto, and by an analysis of the data obtained we were able to calculate the various yields. The following is a summary of these calculations.

Weight of dry corn distilled	73,463,654 lbs.
Gross production of acetone	6,248,131 "
Net " " "	5,741,273 "
Total " " alcohols	12,660,834 "
Gross yield of acetone on dry corn distilled	8.5 per cent.
Net " " " " "	7.76 " "
Yield of alcohols on dry corn distilled	17.23 " "

These results indicate that as the culture and methods of working have improved the percentage yields have increased.

During the distillation it was discovered that ethyl alcohol occurs in the middle fractions. In addition to the large low and high boiling fractions, containing acetone and butyl alcohol respectively, a large fraction is obtained boiling between 85–91.5°C. The latter contains the three neutral products of the fermentation and water. By redistillation pure ethyl alcohol, boiling point 78°C., has been prepared from it. The chemical staff engaged in the work has estimated that the total weight of alcohol produced contains approximately 7.6 per cent of ethyl alcohol.

During the fermentation large volumes of carbon dioxide and hydrogen are produced. The sugar and organic acids formed during the fermentation have been isolated and identified. An attempt has also been made to show the part played by these compounds in the biochemical system underlying the production of the two alcohols and acetone.

Hydrolysis of Starch.

The medium used was in the form of a starch gel, with a heavier layer at the base composed of fiber and protein-containing tissue. Immediately after inoculation liquefaction of the starch com-

mences. By the usual methods it was found that with the liquefaction there is a rapid formation of a reducing sugar. If a small volume of the clear fluid is removed from the flask and added to a 1 per cent starch paste with a slight amount of toluene, it is found that after 24 hours the starch is completely hydrolyzed to a sugar which is a strong reducer of Fehling's solution and forms an osazone very rapidly. From these results and those from suitable controls it was concluded that an enzyme is secreted by the organism which acts upon the starch gel.

The osazone produced was isolated and purified. Only crystals having the characteristic form of glucosazone were to be seen in the specimens obtained from many experiments of this nature. The crystals were found to melt at 204.5°C.

Larger volumes of mash were then inoculated and at the end of 4 hours the flasks were removed from the incubator and an excess of toluene was added to the contents. These were dialyzed against distilled H_2O or Ringer's fluid for 6 days. A mixture of sugar and protein cleavage products passed through the membrane. The solution was evaporated to a small volume under reduced pressure and decolorized with animal charcoal. The residue was extracted with 95 per cent alcohol and reconcentrated. The 95 per cent alcohol solution was then allowed to evaporate at 35°C. A mass of needle-shaped crystals was obtained. The aqueous solution of these crystals was dextrorotatory. From these facts we conclude that the first stage in the fermentation is the hydrolysis of starch to glucose. The nature and properties of the enzyme responsible for this conversion are being more fully investigated.

Acidity Changes.

To obtain information regarding the acidity changes in the fermenting mash, samples of the medium uniform in volume were titrated at regular intervals with 0.1 N NaOH. Curve A in Chart 1 expresses such changes in a normal fermentation. By means of these results the fermentation period can be divided into three sections: (*a*) the time during which the acidity is rising to a maximum, followed by (*b*) the time during which the acidity is falling, and (*c*) a third period during which the acidity rises very slowly from the minimum reached in (*b*).

Curve B in the same chart shows the rate of gas production of this fermentation, based on hourly readings of the rate. We find that during the first of the three phases of the fermentation period the rate rises steadily. When the acidity of the mash is approaching and at the maximum, the rate of gas production falls

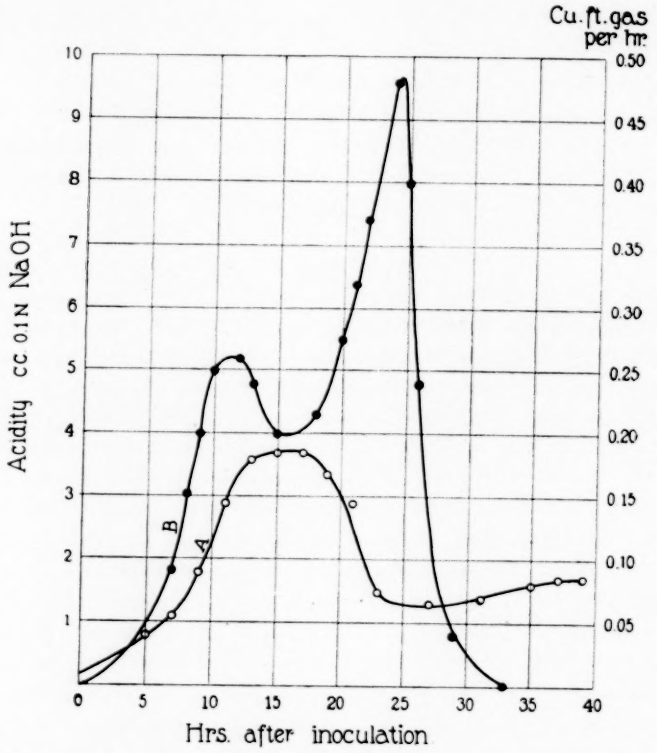

CHART 1. Curve A represents the acidity of 10 cc. samples in terms of 0.1 N NaOH; Curve B the gas produced per hour during the fermentation.

temporarily, and during the second phase rises once more very rapidly to a maximum. Towards the close of the fermentation the rate falls sharply to zero.

The acidity changes and gas production have been studied in two types of abnormal fermentations. For reasons which are

often difficult to define, the life cycle period of the bacillus is sometimes much prolonged. The fermentation is sluggish and the yields are poor. In addition to the morphological features of the organism, such a fermentation has characteristic acidity and gas rate curves. In Chart 2 examples are shown. Curves A and B represent the acidity and rate of gas production readings of a

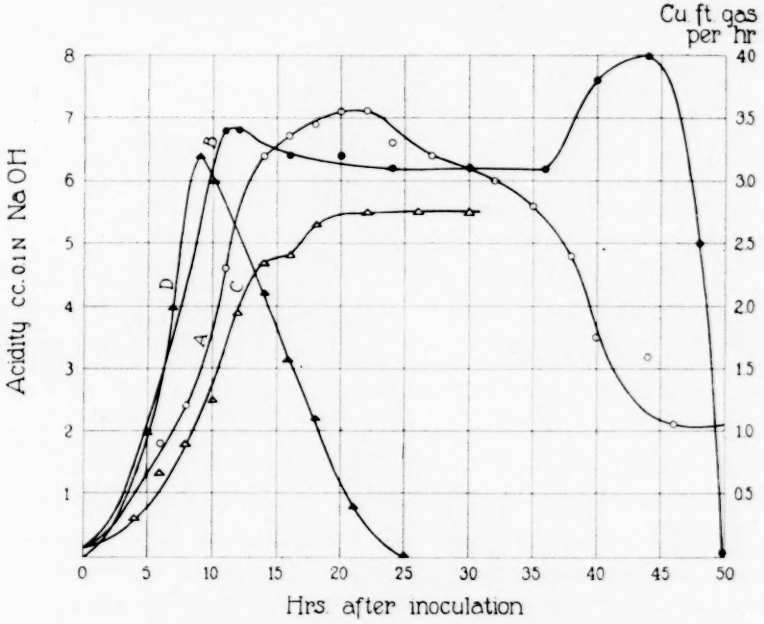

CHART 2. Curve A is the acidity curve and Curve B the gas curve of a slow fermentation. Curves C and D are the corresponding curves for a fermentation conducted at 110° F.

fermentation, volume 20,000 gallons, which was inoculated on September 28, 1917.

With the abnormal prolongation of the maximum acidity period, there is a long delay in the occurrence of the rapid rise in the rate of gas production, and when this increase does develop the maximum rate is very much below the normal.

In the same chart Curves C and D relate to an experiment in which the fermentation was conducted at 110° F. instead of 98° F.

Experiment 1.—A mixture of 3.5 gallons of water and 2 pounds of meal was made in a copper culture vessel. The vessel was maintained at 7 to 8 pounds steam pressure for 3 hours, and the contents were then cooled to the temperature required. To the mash 300 cc. of an active culture were then added. The acidity was determined at regular intervals and the gas produced measured on a small experimental gas-meter. The results obtained are presented in Table I.

TABLE I.

Date.	Time after inoculation.	Gas per hr.	0.1 N NaOH required for 10 cc. sample.
1917	*hrs.*	*cu. ft.*	*cc.*
Nov. 30, 2.00 p.m.			
6.00 "	4	0.140	0.9
8.00 "	6	0.290	
12.00 m.	10	0.445	2.4
Dec. 1, 2.00 a.m.	12	0.379	3.9
4.00 "	14	0.320	4.6
6.00 "	16	0.244	5.1
8.00 "	18	0.161	5.1
12.00 n.	22	0.026	5.0
2.00 p.m.	24	0.012	5.5
4.00 "	26	0.007	5.4
6.00 "	28	0.004	5.6
8.00 "	30	0.002	5.4
10.00 "	32		5.4

We find that working under the conditions mentioned, it is possible to obtain the following results: (*a*) the rate of gas production does not rise after the preliminary fall which has already been observed in the normal, but continues to fall to 0 and (*b*) the acidity does not fall from the normal maximum, but after a slight pause a second increase occurs, and finally an abnormal and constant maximum is reached.

These examples of three different types of fermentation indicate that in the biochemical system there is a close association between the activity of the living cell, expressed by the evolution of gas, and the changes in the acidity of the medium. We may conclude that the fall in acidity during the second phase of the fermentation period is dependent upon organized cell life processes and is not the result of free enzyme activity.

Influence of Starch Concentration on the Acidity Changes of the Medium.

The following are examples of a series of experiments which was carried out to determine in what way the acidity of the fermenting mash was influenced by increasing the concentration of solids. The concentrations used were within the range in which the rate of fermentation is proportional to the concentration of starch in the medium.

Experiment 2.—Three flasks containing maize mash of the concentrations 3, 5, and 7 per cent were sterilized in the autoclave for 2.5 hours at 10 pounds steam pressure. They were inoculated with test-tube cultures of the bacillus in 5 per cent mash. The acidity of the flasks was determined at regular intervals, and the results from the experiment are given in Table II.

TABLE II.

Meal.	Acidity after inoculation. 0.1 N NaOH required for 10 cc. sample.						
	14.5 hrs.	16.5 hrs.	19.5 hrs.	21.5 hrs.	26.5 hrs.	38.5 hrs.	43.5 hrs.
per cent	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
3	3.20	3.90	4.20	4.10	2.60	2.20	2.00
5	3.95	4.95	5.80	5.90	5.00	1.90	2.30
7	4.00	5.60	6.60	6.60	5.70	1.95	2.30

Experiment 3.—The last experiment was repeated with the exception that the flasks fermented contained 2, 4, 6, and 8 per cent mash respectively. In connection with these experiments it is necessary to emphasize the importance of a correct choice of culture with which to inoculate the flasks. It must all be derived from the same spore stock, and the tubes, containing equal volumes, must all be of the same generation and the same age. The results from this experiment are given in Table III.

TABLE III.

Meal.	Acidity after inoculation. 0.1 N NaOH required for 10 cc. sample.							
	7 hrs.	12 hrs.	15 hrs.	20 hrs.	24 hrs.	34 hrs.	40.5 hrs.	60 hrs.
per cent	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
2	0.9	1.8	2.2	2.8	2.7	1.8	1.8	2.0
4	1.2	2.7	3.5	4.2	3.6	2.0	2.0	
6	1.5	3.3	4.5	4.8	4.5	3.1	1.8	
8	1.9	3.6	5.0	5.3	4.9	2.6	1.9	

These results indicate that the acidity of the mash during the fermentation is controlled at least in part by the concentration of meal used. We find that in each series of flasks the acidity increased with the concentration. The two series of flasks when grouped together do not form a regular sequence as regards both the concentration of the mash and the acidity readings. For example, the 3 per cent flask of the first set does not occupy a position between the 2 and 4 per cent flasks of the second set. This condition of things has been confirmed during a much wider study of fermentations of various concentrations. It is clear that the influence of the concentration of starch in the medium on the acidity must be regarded as one only of several factors affecting the latter.

Influence of the Rate of Fermentation on the Maximum Acidity.

Until the biochemistry of the fermentation is more completely analyzed and understood we can only group together a number of these unknown factors and express the sum of their influences in general terms, such as the time occupied by the fermentation of a known weight of meal, or the yields of the various products under certain conditions. In the production of acetone on a large scale, when the time factor was of great importance, we expected those fermentations which had a maximum acidity higher than the average to occupy a longer time period; those with a lower maximum to be correspondingly quicker. To verify such a hypothesis the accumulated observations of a large number of carefully controlled experiments will be required. At present there are available the laboratory records of several thousands of fermentations, showing the volume and concentration of the medium, the time occupied by, the gas produced by, and the acidity changes of the fermentation. They were obtained originally for practical purposes, but although not free from errors they may assist in the consideration of this problem. One of my colleagues has taken from the records several hundreds of complete sets of observations relating to large fermentations of uniform volume and concentration. These have been arranged in groups according to the number of hours in the fermentation period. The number of examples in each and the average maximum acidity

TABLE IV.

Time of fermentation.	No. of examples.	Average maximum acidity.	Time of fermentation.	No. of examples.	Average maximum acidity.
hrs.		*cc.*	*hrs.*		*cc.*
24	9	5.1	35	35	5.8
25	16	5.5	36	36	5.65
26	34	5.06	37	30	5.87
27	35	5.3	38	28	5.9
28	70	5.46	39	13	5.6
29	92	5.5	40	18	5.9
30	81	5.46	41	16	5.9
31	68	5.7	42	10	6.1
32	59	5.6	43	7	6.2
33	60	5.7	44	3	6.3
34	54	5.76			

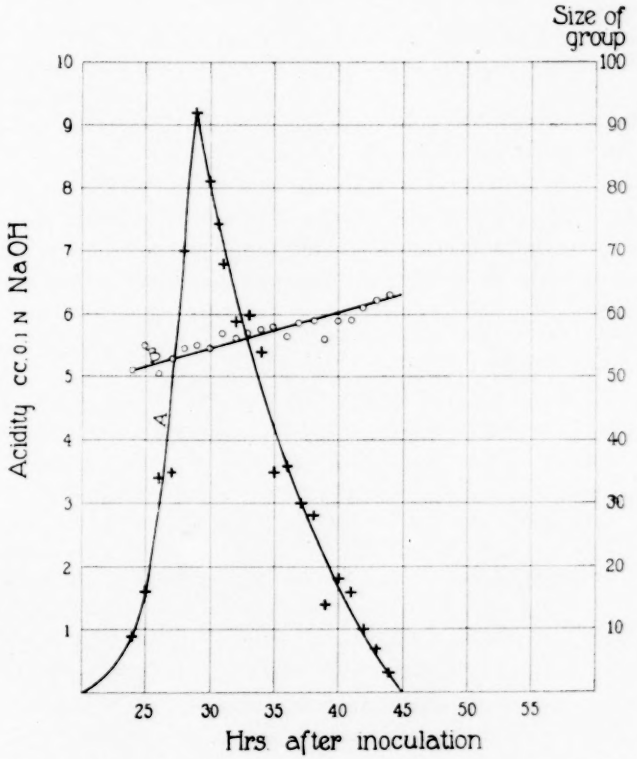

CHART 3. The curves are based on the figures contained in Table IV.

of the groups were then calculated. These results are given in Table IV. The acidity readings are for 10 cc. samples in terms of 0.1 N NaOH.

These results are represented by curves in Chart 3. Curve B shows the maximum acidity readings, and furnishes evidence in support of the view that the rate of the fermentation and the acidity of the medium are correlated. Curve A represents the number of examples in each group, and is thus a normal distribution curve of fermentations for the time occupied. These figures were calculated for the period from the time of inoculation to the end of fermentation. For a period of approximately 8 hours the filling of the tank was in progress, and therefore these figures cannot be compared directly with those relating to fermentations of a known volume of medium, inoculated at a given time and afterwards allowed to ferment undisturbed.

Isolation and Identification of Volatile Organic Acids.

The following is a description of the methods used on three occasions when the acids have been obtained and identified. Batches of 50 gallons of mash were prepared, sterilized, and fermented in a copper vessel in the laboratory. During the early stages of the fermentation the acidity of the mash was determined at regular intervals. When successive readings indicated that the acidity was approaching the maximum, the vessel was connected to an adjacent water-cooled, coil condenser. The contents were quickly raised to boiling point, and about 13 gallons of distillate collected in large glass vessels. The whole distillate was saturated with common salt, and extracted with 1,500 cc. of ether. After separating, the ether-soluble fraction was filtered and the ether partially removed by distillation from a water bath maintained below 40°C. The mixture of neutral and acid products of the fermentation was diluted with an equal volume of distilled water. The amount of acid present was estimated in terms of butyric acid and the mixture was then completely neutralized with twice the required amount of calcium carbonate. After an interval of several hours the excess carbonate was removed by filtration under pressure, and was then washed with distilled water. The combined solutions of calcium salts were

evaporated to dryness on the steam bath, and the residues obtained were more thoroughly freed from traces of the neutral compounds by dry heat in the oven, maintained at 100°C. The dry salts were weighed and the amount of sulfuric acid required to liberate the organic acids was calculated. An excess of dilute sulfuric was then added to the salts. The mixture was extracted with two batches of ether, and the united portions of ether-soluble material were distilled in the following manner.

The ether was removed on the water bath maintained at temperatures below 40°C. The fractions of the remainder which came over below 130°C. were removed at atmospheric pressure, and the residue was distilled under reduced pressure. The following results are from the records of these experiments.

Experiment 4.—

	A.	B.
Calcium salts obtained	150 gm.	112 gm.
Mixture of acids distilled	80.00 cc.	88.00 cc.

Distillation 1.

Fraction:

	A. cc.	B. cc.
(*a*) Below 40°C	0.00	15.3
(*b*) 40–70°C	0.00	0.00
(*c*) 70–105°C	0.00	1.40
(*d*) 105–130°C	11.00	12.70
(*e*) Above 130°C	67.00	55.00

Practically the whole 105–130°C. fraction came over between 117–122°C.

Distillation 2.

The residues boiling above 130°C., *i.e.* Fraction (*e*) from Distillation 1, were combined and fractionally distilled under reduced pressure. The whole of this batch, with the exception of a small residue, came over while the thermometer fluctuated between 77–79°C. The corrected boiling point of the fraction was found to be 163.3°C.

Esterification.—The combined fractions for the temperature interval 105–130°C. amounted to 22 cc. when recovered from the containers. An equal volume of ethyl alcohol and 1 cc. of concentrated H_2SO_4 were added to the fraction, and the mixture was digested for 3 hours in a small flask below a spiral condenser. After cooling under the tap the products of the digestion were neutralized with successive washings of dilute sodium carbonate. The ester layer was separated and shaken with a small volume of distilled water containing an excess of calcium chloride. The

ester layer was again separated and placed over anhydrous calcium chloride for 24 hours. The upper layer, free from water and traces of alcohol, was then distilled at atmospheric pressure.

Distillation 3.

Volume of ester layer distilled	21.60 cc.
Fraction 74–80°C.	14.30 "
Residue	4.40 "
Summary of results from Experiment 4.	
Temperature interval of first large fraction	117–122°C.
(B.P. of acetic acid)	118 "
Temperature interval covered during the distillation of the ethyl ester prepared from this fraction	74–80 "
(B.P. of ethyl acetate)	77 "
Corrected B.P. of the fraction obtained from D2	163.3 "
(B.P. of N butyric acid)	163.0 "

These results indicate that during the fermentation acetic acid and a larger quantity of N butyric acid are formed. There remains however the possibility that smaller amounts of one or more other acids are formed. Although the experiments described were conducted with care, and as far as possible loss of material was avoided during the various stages, it is not considered advisable to draw any quantitative conclusions from the results obtained. They confirm the results of investigations by Henley (4) and his collaborators, who have also obtained results of a quantitative character. Our major problem has now been simplified and can be stated in the following terms. What is the relation, in the biochemical system of the fermentation, between acetic and N butyric acids and the neutral products of the fermentation, acetone and the two alcohols, ethyl and butyl?

Relation between the Formation of the Neutral Products and Changes in the Acidity of the Medium.

The following experiments were performed to find out the relation in time between the changing acidity of the mash and the rate of the formation of the neutral products.

Experiment 5.—25 pounds of meal were added to approximately 40 gallons of cold water, in a large copper fermentation vessel. The mixture was well stirred by means of a motor-driven apparatus, and the vessel contents were slowly raised to boiling point by means of live steam. The mash so

prepared was then sterilized in the same closed vessel for 3 hours at 7 to 8 pounds steam pressure. At the close of this period the volume of the batch was approximately 66 gallons, and after cooling the mash it contained roughly 4 per cent of solids. The culture added was 6 liters of an active culture of the bacillus in 5 per cent maize mash. During the fermentation the temperature of the vessel was maintained at 98°F. After 10 hours of the fermentation period the acidity of medium was determined at regular intervals. Samples, 150 cc. in volume, were withdrawn at intervals of 2 hours and distilled. The distillate collected from each of these was 50 cc. in volume and was made up accurately to 100 cc. with distilled water. The acetone content of each sample was determined by titration, using the Messinger method. The results obtained from this experiment are presented in Table V.

TABLE V.

Date.	Time after inoculation.	0.1 N NaOH required for 10 cc. sample.	Acetone in 150 cc. sample.
1919	*hrs.*	*cc.*	*gm.*
Apr. 9, 10.00 a.m.	10	1.15	0.005
12.00 n.	12	1.80	0.004
2.00 p.m.	14	2.65	0.005
4.00 "	16	3.71	0.008
6.00 "	18	4.36	0.010
8.00 "	20	5.06	0.021
10.00 "	22	5.59	0.049
11.30 "	23.5	5.60	
May 9, 9.00 a.m.	33	3.82	
10.00 "	34	3.74	0.256
12.00 n.	36	3.62	0.274
2.00 p.m.	38	3.19	0.317
4.00 "	40	3.15	0.309
6.00 "	42	2.90	0.326
8.00 "	44	2.85	0.331
June 9, 10.00 a.m.	58	3.13	0.356
12.00 n.	60		0.368

Experiment 6.—The last experiment was repeated with the exception that an effort was made to obtain results showing the rate of the formation of alcohols. The same tests were made as in Experiment 5 and in addition a number of larger samples, 2,000 cc. in volume, were distilled at regular intervals of time during the fermentation. About 300 cc. of distillate were collected from each one and redistilled from a 500 cc. flask, using as a still-head a three-section Young's fractionating column. The distillate from each sample was completely saturated with K_2CO_3 and allowed to stand over an excess for 60 hours in the refrigerator. The oil layer from each sample was separated and measured by volume. By the method

previously described the weight of acetone, and therefore the volume, present in the 2,000 cc. samples was estimated. From these two figures it is possible to obtain, by difference, the volume of the mixture of the two alcohols in the total volume of neutral products in the sample. Owing to the presence of water in the oil the results so obtained do not represent dry, pure alcohol. The amount of water is not considerable and the results, in spite of this error, do give us the information required; *i.e.*, the relation between the rate of formation of the alcohols and the changes in acidity of the medium. The results of this experiment are given in Table VI.

TABLE VI.

Date.	Time after inoculation.	0.1 N NaOH required for 10 cc. sample.	Acetone per 150 cc.	Acetone per 2,000 cc.	Total oil per 2,000 cc.	Total alcohol per 3,000 cc.
1919	*hrs.*	*cc.*	*gm.*	*cc.*	*cc.*	*cc.*
Sept. 16, 9.30 a.m.	11	2.60	0.010	0.30	0.60	0.30
11.30 "	13	3.95	0.024	0.40	0.60	0.20
1.30 p.m.	15	4.80	0.040	0.66	1.00	0.34
3.30 "	17	5.39	0.068	1.00	2.50	1.50
5.30 "	19	5.33	0.111	1.85	3.60	1.75
7.30 "	21	4.90	0.174	2.90	7.60	3.70
9.30 "	23	4.25	0.243	4.05	10.80	6.75
10.30 "	24	3.94				
Sept. 17, 9.30 a.m.	35	1.85	0.616	10.27	31.00	20.73
11.30 "	37	2.06	0.628	10.47	33.50	23.03
1.30 p.m.	39	2.07	0.656	10.94	Lost.	
3.30 "	41	2.09	0.655	10.92	35.00	24.08
5.30 "	43	2.10	0.675	11.25	35.00	23.75
7.30 "	45	2.20	0.663*	11.05	35.00	23.95
Sept. 18, 9.30 a.m.	59	2.31	0.673	11.22	35.50	22.28
1.30 p.m.	63	2.35	0.652*	10.87	Lost.	

* Acetone low on account of loss during distillation. The results obtained in Experiment 6 are represented in the form of curves in Chart 4.

The curves in Chart 4 show that during the first phase of the fermentation period, when the hydrolysis of starch and the increase in the acidity of the medium are in progress, there is little formation of the alcohols or acetone. Towards the close of this phase formation commences at a slow rate. During the second phase, when we find a diminution in the amount of free acid, the neutral compounds are produced with great rapidity. This is also the time during which we have already observed a marked increase in the rate of gas production. It is interesting also to

compare the relative amounts of acetone and the alcohols in the early samples analyzed with the amounts in the final sample. We find that the early ones contain more acetone than alcohol; whereas the final sample contains approximately twice as much alcohol as acetone. These facts suggest that the formation of

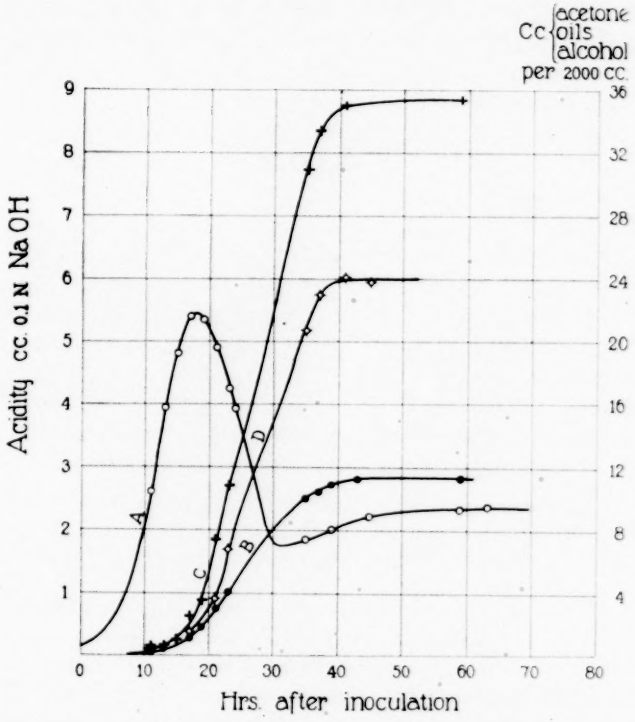

CHART 4. Curve A is the acidity curve and Curves B, C, and D the rate of production curves of acetone, total neutral products (oils), and alcohols respectively of the fermentation described in Experiment 6.

acetone commences slightly earlier than that of the alcohols. During the final phase of the fermentation, when the acidity of the medium rises very slowly to a constant, there is a very pronounced fall in the rate of production of acetone and alcohol until the end of the fermentation.

Acidity Changes during the Fermentation of Mash Previously Acidified with Various Organic Acids.

A large number of experiments have been performed in which small volumes of organic acid have been added to the medium at different points in the fermentation period. For example, varying amounts of butyric acid were added to a number of flask cultures at a point when the acidity of the medium was falling rapidly. In a typical case an increase in acidity equivalent to 1.1 cc. of 0.1 N NaOH per 10 cc. of mash was brought about. This addition had no harmful effect on the culture, and, what is equally significant, the final acidity of the flask was normal, 10 cc. = 2.3 cc. of 0.1 N NaOH.

TABLE VII.

Date.	Time after inoculation.*	Cc. 0.1 N NaOH required for 10 cc. sample.		
		Control flask.	Acetic flask.	Butyric flask.
1918	*hrs.*	*cc.*	*cc.*	*cc.*
Jan. 16, 5.00 p.m.	0		3.60	3.50
" 17, 10.30 a.m.	17.5	3.60	4.00	3.40
12.00 n.	19	4.00	4.40	3.90
4.00 p.m.	23	3.60	5.00	4.30
Jan. 18, 10.00 a.m.	41	2.00	2.30	3.00
12.00 n.	43	2.20	2.20	2.30

* The flasks were inoculated at 5.00 p.m., Jan. 16, 1918.

In a second group of experiments the acid was added before inoculation. The acidity changes of each flask were followed, and as the experiment was repeated the initial acidity of the medium, due to free organic acid, was increased. Finally sufficient acid had been added to inhibit the life processes of the bacillus. In other flasks with a lower initial acidity, the fermentation was only retarded. The following is a description in greater detail of such a series of flasks.

Experiment 7.—Three flasks each containing 750 cc. of 5 per cent maize mash were sterilized for 2.5 hours at 10 pounds steam pressure. Flask A acted as a control, Flask B was acidified with acetic acid, and Flask C was acidified with butyric acid. The three flasks were inoculated at the same time with equal amounts of culture and were incubated together. The acidity of the flasks was determined at intervals. At the close of the

fermentation the flasks were similar in external appearance. The starch was completely hydrolyzed and the flasks contained clear yellowish green fluid upon which the slimy albuminous residue was floating. The results from this experiment are presented in Table VII.

The results show that during the fermentation of Flasks A and B a portion at least of the organic acid added to the flasks was involved in some biochemical reaction with the result that it was converted into some essentially different substance. Similar results were obtained when a sufficient amount of propionic acid was added to the mash previous to inoculation. An effort was then made to isolate and identify the compounds produced by the reaction involving the organic acids.

Quantitative Experiments Showing the Compounds Produced during the Fermentation of Mash Plus Various Organic Acids.

The object of the experiment was to determine the nature and volume of the neutral products of fermentations of maize mash to which known quantities of acetic, propionic, or butyric acid have been added previous to inoculation. The general method adopted was to ferment flasks of medium of known volume and concentration, to which the acid was added, and at the close of the fermentation to isolate, measure, and identify the various compounds by careful fractional distillation. The flasks were arranged in groups according to the nature of the organic acid added to the mash.

Experiment 8.—Group A.—10 liters of mash were prepared containing 400 gm. of maize meal. The mash was sterilized for 2.5 hours in the autoclave at 10 pounds steam pressure. This group was the control for the experiment.

Group B.—Eight flasks containing an equal volume of mash of the same concentration, and sterilized in the same manner. To each flask 2.5 cc. of glacial acetic acid were added previous to inoculation.

Group C.—Eight flasks similar to those in Group A, with 3.0 cc. of propionic acid added to each flask before inoculation.

Group D.—Eight flasks similar to the above, to which 3.0 cc. of butyric acid were added.

The acid was added when the flasks had cooled, and they were then inoculated, each with 50 cc. of an active culture of the bacillus in 5 per cent mash, used 20 hours after inoculation. The flasks of the four groups were incubated at 36.5°C., and during the fermentation period they were

not disturbed in any way. In all cases the mash was completely fermented. The contents of the different groups of flasks were then examined in the following manner.

Distillation 1 (D1).—The 10 liters of beer from each group were distilled from a large copper vessel. The distillate was condensed in a straight glass tube condenser, and was collected until it no longer contained traces of butyl alcohol. Approximately 1,500 cc. were obtained from each group.

Distillation 2 (D2).—The distillate from D1 of each group was partially saturated with NaCl and redistilled from a flask. A three-section Young's fractionating column was used for a still-head. The distillation was continued in each case until all the butyl had passed over.

Distillation 3 (D3).—The second distillation was repeated using a smaller flask. The fractions boiling above and below 75°C. were collected in different receivers. The higher boiling fraction was shaken with a con-

TABLE VIII.

Temperature interval.	Volume of fraction.			
	Control.	Acetic group.	Propionic group.	Butyric group.
°C.	cc.	cc.	cc.	cc.
Below 65.	24.00	33.75	32.50	29.50
65–75	6.00	9.75	7.00	3.50
75–85	6.00	5.00	9.75	10.00
85–90.5	11.00	9.50	13.00	8.00
90.5–105	4.00	6.25	4.75	5.00
105–115	8.00	8.00	23.00	12.50
Above 115.	58.00	59.00	49.00	72.50
Total volume	117.00	131.25	139.00	141.00
Volume of acid added to mash.	0.00	20.00	24.00	24.00

siderable amount of K_2CO_3 and allowed to stand over an excess for 24 hours. The upper layer was separated from the aqueous layer and restored to the lower boiling fraction. The combined fractions were redistilled.

Distillation 4 (D4).—For this distillation a smaller column, made up of three bulbs each containing one loose, blown glass bead, was used. The temperature intervals adopted and the volumes of the fractions obtained for the four groups of flasks are embodied in Table VIII.

The small residue is in each case included in the final fraction. From the relatively small volume of the fraction obtained between 90.5–105°C. in each case we may conclude that the amount of water present was very small, and from the uniformity of the fractions from the four series that the amount still present in the

distillates was approximately the same in all cases. In the three series of flasks to which the acids were added previous to inoculation there was a considerable increase in the volume of neutral products of the fermentation. When the results of the experiment are considered in greater detail we find that (*a*) in the acetic series there was an increase of 45 per cent in the products boiling below 75°C., and smaller differences in the higher fractions when compared with those obtained from the control; (*b*) in the propionic series there was an increase of 31.7 per cent in the fractions boiling below 75°C., an increase of 187.5 per cent in the fraction boiling between 105–115°C., and a decrease in the fraction boiling above 115°C. of 15.5 per cent; and (*c*) in the butyric series there was an increase of 10 per cent in the fractions boiling below 75°C., and an increase of 25 per cent in the fraction boiling above 115°C. The results obtained from this experiment were verified three times before proceeding further with the research. An effort was then made to elucidate the nature of the compounds produced from the propionic acid series.

From the results obtained in the butyric acid series it was clear that there was a large increase in the production of butyl alcohol. There seemed to be strong evidence for the assumption that this was produced during the fermentation from the acid added to the mash. If propyl alcohol were produced from the acid added to the third series, in what fractions would it appear in the final distillation? In this connection it was recalled that of the ethyl alcohol produced during the normal fermentation the majority is found with butyl alcohol and water in the 85–91°C. fraction. Using pure propyl and butyl alcohols it was then found what results were obtained when a mixture of the two was fractionally distilled.

Experiment 9.—(*A*). A known volume of butyl alcohol was distilled using the apparatus which had been used in D3 and D4 of Experiment 8. The following figures relate to this distillation.

	cc.	cc.
Volume of butyl alcohol distilled	75.00	60.00
Fraction boiling below 105°C.	0.00	0.00
" " from 105–115°C.	3.00	4.50
" " above 115°C.	72.50	55.50

(*B*). A mixture of the pure alcohols, with butyl alcohol in excess, was then distilled.

	cc.	cc.
Volume of butyl in the mixture	75.00	60.00
" " propyl " " "	5.00	5.00
Fraction boiling below 105°C.	1.00	0.00
" " from 105–115°C.	15.00	17.00

From these results it was concluded that when such a mixture of the two alcohols is distilled a large fraction containing both alcohols comes over between 105–115°C.

Experiment 10.—The object of this experiment was to obtain a larger volume of the unknown fraction in the products from the mash plus propionic acid fermentation. Mash was prepared containing 2,000 gm. of meal and was then made up to 50 liters. After sterilization the mash was allowed to cool and 120 cc. of propionic acid were added. The same per-

TABLE IX.

Temperature interval.	Volume of fraction.		Temperature interval.
	Experiment 10.*	Group 3, Experiment 8.	
°C.	cc.	cc.	°C.
57–65	197.00	32.50	Below 65.
65–75	12.00	7.00	65–75
75–85	35.00	9.75	75–85
85–95	26.00	13.00	85–90.5
95–105	47.00	4.75	90.5–105
105–115	43.50	23.00	105–115
Above 115.	297.00	49.00	Above 115.
Total volume..	657.50	139.00	

* The amounts used in Experiment 10 were five times those used in Group 3, Experiment 8.

centage of inoculant was added as in the last experiment and the fermentation was allowed to proceed undisturbed in the incubator at 36.5°C. The products of the fermentation were analyzed by distillation in the manner previously described. Traces of water were removed by salting out the alcohol from the 90.5–95°C. fraction. The results obtained from this experiment after the middle fractions had been redistilled several times are given in Table IX. The results obtained from D4, Experiment 8, of flasks containing propionic acid are included for comparative purposes.

The figures indicate that in the redistillation of the middle fractions, as the acetone, butyl alcohol, and water contained in them are eliminated, the relative volumes of these fractions change and progressive increases are observed in the 75–85 and 95–105°C.

fractions. As we have already observed, the accumulation of distillate in the first of these fractions is due to the gradual purification of the ethyl alcohol. The formation of the second of these large fractions was due to the presence of propyl alcohol which was gradually separated from the larger volume of butyl alcohol. From the fractions boiling above 95°C. shown in Table IX, 37.00 cc. of propyl alcohol were obtained.

Discussion of Experiments 8, 9, and 10.

In our consideration of the facts established by a comparison of the nature and volume of the compounds produced during the normal and abnormal fermentations, attention is called only to

TABLE X.

		per cent.	*per cent*
Mash plus 0.2 per cent acetic acid by volume.	Increase in the yield of acetone.....	45.0	
	" " volume of ethyl alcohol in terms of acetic acid added.....		0.00
Mash plus 0.24 per cent propionic acid by volume.	Increase in the yield of acetone.....	30.0	
	" " " " " propyl alcohol in terms of propionic added.....		30.0
Mash plus 0.24 per cent butyric acid by volume.	Increase in the yield of acetone.....	10.0	
	" " " " " butyl alcohol in terms of butyric added.....		80.0

differences regarding the existence of which there does not appear to be room for doubt. With regard to the results obtained from the flasks containing mash plus acetic acid, a slight increase in the yield of butyl alcohol was observed, but further investigation is deemed necessary before this can be regarded as a characteristic result of such an experiment. There remains also the possibility that, working with larger volumes of mash to which a larger percentage of acetic acid has been added before inoculation, an increase in the production of ethyl alcohol might be produced.

Neglecting these possibilities, from the results obtained it has been calculated what the increase in the yield of acetone was in each group of flasks, and approximately what was the ratio between the alcohol equivalent of the amount of the organic acid

added, and the increase in the production of the corresponding alcohol. It was assumed from the results obtained in Experiment 10 that, when 120 cc. of propionic acid are added to 50 liters of mash, 37 cc. of propyl alcohol are formed. This estimate disregards the propyl present in the final 99–115°C. fraction which was too small in volume to refractionate. The figures in Table X regarding the acetic and butyric experiments are based on the results of Experiment 8.

DISCUSSION.

From the foregoing experiments it is clear that the organism when growing in a medium rich in starch secretes an enzyme, or group of enzymes, which hydrolyzes the starch to glucose. The sugar passes into the cell and by its oxidation acetic and n butyric acids are produced.

It is interesting to compare the sugar and the two acids produced with the acids formed during the fermentation of xylose (5). In both cases the sum of the carbon atoms in the two acids is the number of carbon atoms in the sugar from which they are derived.

The acidity of the fermentation gradually rises. At a slightly later stage another process commences; namely, the conversion of the acids into substances which are neutral. Eventually the second process is more active than the first and there is for several hours a flow of acid into the cell from the medium. Towards the end of the fermentation the balance is again reversed and the acidity of the medium rises slightly once more. The method adopted for the determination of the acidity only serves to indicate the balance between the acid-forming and acid-destroying systems and does not furnish data regarding the true acidity of the normal fermentation.

The experiments showing the variations in the acidity of fermenting media of different concentrations, and of abnormal fermentations due to the temperature conditions or the low vitality of the organism, indicate that the formation and destruction of the acids are not independent of but are essential parts of the biochemical system responsible for the formation of the alcohols and acetone. It has been shown that the period during which the destruction of the acids is most active is the period during

which the neutral products are being most rapidly formed. These facts can be explained by the hypothesis that the organic acids are intermediate compounds in the fermentation.

We shall discuss in the first place the evidence supporting the hypothesis that the acids are reduced to the alcohols. When acetic, propionic, and butyric acids were added to different portions of mash, equal in volume and concentration, it was found that there was no change in the yield of ethyl alcohol, that propyl alcohol was formed, and that there was a large increase in the yield of butyl alcohol. Now if the acids give rise to the alcohols in the normal fermentation, why was there no marked increase in the yield of ethyl alcohol when acetic acid was added to the mash? In order to increase the yield of the corresponding alcohol the acid must pass through the cell wall. What are the factors present under the conditions obtaining, which are known to influence the penetration of cell walls by acid? The cells were immersed in solutions of acids, present in varying concentrations. When the flow of acid into the cells started in the acetic flasks the order of concentration was: butyric = acetic; in the propionic flasks: butyric = propionic > acetic; and in the butyric flasks: butyric > acetic. Crozier (6) has shown, working with animal cells and 0.01 N solutions of the fatty acids, that the order of penetration among the lower members of the series is as follows: valeric > butyric > propionic > acetic. Harvey (7) has obtained similar results, also working with animal cells. Haas (8) arrived at a similar conclusion from experiments on the penetration of living plant cells by 0.01 N solutions, made by titration, of acids and alkalies. Very different results were obtained when the acid solutions were standardized by the gas chain method. If the same law of penetration applies when bacteria are used, and if the acid passing through the wall is converted to the corresponding alcohol, then the increases in the yields of the alcohols, expressed as ratios of the alcohol equivalents of the amounts of the different acids added to the mash, should correspond to the order of penetration. The figures in the right hand column of Table X do so in a very striking manner. Reilly (4) has observed that in the later part of the fermentation, *i.e.* when acid is passing into the cell, the percentage of acetic acid in the total free acid increases. The results of these experiments show that during the normal fermentation the two acids are reduced to the corresponding alcohols.

We shall now consider the results in relation to the production of acetone. We observe that during the rapid destruction of the acids formed during the normal fermentation there is a corresponding production of acetone, and that without the first the second does not occur. Is it possible that acetone is also formed from one or both of the acids? From the flasks to which various acids were added largely increased yields of acetone were obtained. Reilly (4) has made similar observations in connection with acetic acid. He states:

> "It appeared that the bacteria or enzymes were actually able to reduce the acetic acidity in considerable quantities. The percentage of acetone was increased by the addition of acetic acid. If acetic acid were made by an ordinary fermentation process, for example, by souring cheap wines, the dilute solution could be pumped directly into the fermentation vat for conversion into acetone."

Our results show that if we assume a conversion of acetic acid into acetone, we must in the end assume the presence in the cell of some system by which acetone is formed from the three acids, acetic, propionic, and butyric. This is highly improbable. Furthermore if the acid passing through the cell wall is partially converted into acetone we should expect the largest increase in yield from the butyric acid flasks, for, as we have observed earlier in the discussion, the acids penetrate the cell wall in increasing amounts as we ascend the series. It was found that the increase in the yield of acetone diminishes in a regular manner as we ascend the series. For these reasons I conclude that the yield of acetone is affected by influences exerted by an acid on intercellular life by virtue of its properties and presence in the surrounding solution only and not by conversion into acetone within the cell.

Although the acetone is not produced from acid which passes from the medium into the cell, is it formed from organic acid which flows directly from the acid-producing to the acetone-producing region in the cell. It is also necessary to assume that the acid passing into the cell does not reach the acetone system. Such an organization might be influenced in the manner described by the presence of large quantities of acid in the mash before inoculation, for example, by restricting the flow of acid from the cell. For theoretical reasons it does not seem probable that acetone is produced from acetic acid, but there remains the possibility with

regard to butyric acid. Witzemann (9) has shown that acetone is produced by the oxidation of butyric acid by means of hydrogen peroxide. It would seem advisable however to leave this portion of our problem for further investigation.

CONCLUSIONS.

From the results obtained by this investigation we conclude that:

1. *Bacillus granulobacter pectinovorum* growing in a medium rich in starch changes the latter into glucose by exoenzyme activity.
2. Glucose passes into the cell and is oxidized to acetic and butyric acids.
3. These acids are in part reduced to the corresponding alcohols.

I wish to thank my colleague Mr. A. M. Wynne for his assistance during the prosecution of this research.

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DETERMINATION OF CHLORIDES IN WHOLE BLOOD.

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(Received for publication, January 26, 1920.)

As it is desirable at times to determine the chloride content of the whole blood rather than of the plasma only, the applicability to this purpose of the Van Slyke-Donleavy method[1] for the determination of plasma chlorides was investigated.

It was found that some component of the laked cells other than the chloride has the property of binding silver, so that the direct application of the Van Slyke-Donleavy method to whole blood gives readings too high by 30 to 40 per cent. If, however, after laking, the protein is precipitated by picric acid alone, or by picric and nitric acids, and the protein-free filtrate is treated with silver nitrate, a quantitative precipitation of the total chloride of the whole blood is obtained and the final titration may be carried out as in the Van Slyke-Donleavy method. The accuracy of this modification was tested on whole oxalated ox blood by comparison with chloride estimation by the Carius method, using the technique for destroying the organic matter adopted by Vinograd[2] and a Volhard titration of the silver remaining unbound.

Carius Method.

Whole oxalated ox blood was analyzed for chloride content by the Carius method as follows.

Approximately 1 cc. of the whole blood was introduced from a pipette into the bottom of a bomb tube and the amount accurately determined by weighing the pipette before and after delivery.

[1] Van Slyke, D. D., and Donleavy, J. J., *J. Biol. Chem.*, 1919, xxxvii, 551.

[2] Vinograd, M., *J. Am. Chem. Soc.*, 1914, xxxvi, 1548, also *Studies from The Rockefeller Institute for Medical Research*, 1915, xxii, 372.

1 cc. of a solution of silver nitrate containing 20.4 mg. of $AgNO_3$ per cc. was carefully introduced into the bottom of the tube. The tube was then immersed in a water bath at 100° and the moisture-laden air in the upper portion of the tube continually removed by aspiration until the water was driven from the mixture of blood and silver nitrate. 1 cc. of fuming nitric acid in a small tube was then introduced into the tube. The tube after being sealed was slowly heated to 180° and this temperature maintained for 3 hours. After cooling, opening, and washing out the colorless solution with distilled water the remaining unprecipitated silver was titrated by Volhard's method with a 0.015 M solution of NH_4CNS, the chloride obtained by difference being expressed as NaCl. The results of four determinations are given in Table I.

Results with Modified Van Slyke-Donleavy Method.

Into each of two 50 cc. volumetric flasks were introduced 4 cc. of the same whole oxalated ox blood used in the Carius analysis and the weight of blood introduced was determined by weighing the flasks before and after introduction of the blood. After laking by addition of 20 cc. of distilled water, there were added 20 cc. of the following solution of nitric and picric acids:

	cc.
HNO_3 (sp. gr. 1.42)	250.0
Picric acid	7.5
Distilled water to	1,000.0

The flask was then filled to the mark with distilled water and repeatedly inverted. After allowing 10 minutes for complete precipitation of the protein, the mixture was filtered through a dry filter and 25 cc. of the clear yellow filtrate were introduced into a 50 cc. volumetric flask. To this were added 10 cc. of an M/29.25 $AgNO_3$ solution, the flask was filled to the mark, and two drops of caprylic alcohol were added. After standing over night (to promote clear filtration) the clear supernatant fluid was removed with a pipette, filtered, and two 20 cc. portions were taken. These were titrated with KI solution after addition of 4 cc. of the special starch solution as described in the Van Slyke-Donleavy method.

The results of four determinations are given, together with those on the same blood by the Carius method, in Table I. In the last column are given also the results obtained in attempting to apply the Van Slyke-Donleavy method, without modification, to whole blood.

The chief difficulty encountered in the present method was in securing a clear filtrate after the precipitation of the AgCl. This is probably somewhat facilitated by using more picric acid and

TABLE I.

Results of Chloride Determinations on a Specimen of Whole Blood.

Carius method.			Van Slyke-Donleavy method modified for whole blood by precipitating proteins and AgCl separately.		VanSlyke-Donleavy method for plasma applied to whole blood without modification.
Weight of blood.	NaCl found.	NaCl found per gm. of blood.	Weight of blood.	NaCl found per gm. of blood.	NaCl found per gm. of blood.
gm.	*mg.*	*mg.*	*gm.*	*mg.*	*mg.*
0.998	4.47	4.48	4.108	4.53	6.00
1.000	4.54	4.54		4.53	5.95
1.005	4.57	4.55	4.113	4.54	5.98
1.002	4.57	4.56		4.53	6.05
Average		4.53		4.53	5.99

less nitric acid for the precipitation of the protein, then adding the required amount of nitric acid to the final 20 cc. portions just before addition of the starch solution. The most effective means of securing a clear filtrate, however, is by permitting the solution to stand in the dark over night after the addition of the silver nitrate; a perfectly clear supernatant liquid can then be pipetted from the flask.

The technique finally adopted is as follows.

Method for Determination of Chlorides in Whole Blood.

Take 3 cc. of blood with 15 cc. of water in a 60 cc. flask. Add 30 cc. of saturated picric acid solution and sufficient water to bring the volume to 60 cc. (27 cc. of water in all, and 30 cc. of picric acid may be measured from burettes if a 60 cc. measuring flask is not available). The contents of the flask are mixed, and after 10 minutes are filtered.

To 40 cc. of the filtrate 10 cc. of the M/29.25 silver nitrate solution used by McLean and Van Slyke[3] are added, with two drops of caprylic alcohol (the silver solution contains per liter 5.812 gm. of $AgNO_3$ and 250 cc. of HNO_3 (sp. gr. 1.42). The solutions are thoroughly mixed, and preferably allowed to stand over night to allow the AgCl to coagulate and settle. The supernatant solution is decanted through a small filter paper, and 20 cc. are titrated as described by Van Slyke and Donleavy. The calculation also is the same, since the 20 cc. of filtrate titrated in this case, as in the method of Van Slyke and Donleavy, represent 0.8 cc. of the original material, blood or blood plasma.

Attention may be called to the fact that the use of the unmodified Van Slyke-Donleavy method with a plasma that is considerably stained as the result of partial hemolysis of the cells will give values that are unduly high.

CONCLUSIONS.

1. The method described by Van Slyke and Donleavy for the determination of plasma chlorides is not directly applicable to whole or to laked blood.

2. A modified technique for whole or laked blood is described in which the precipitation and removal of the protein precede the addition of the silver.

[3] McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.

EXPERIMENTS ON THE UTILIZATION OF THE CALCIUM OF CARROTS BY MAN.

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(Received for publication, January 24, 1920.)

McClugage and Mendel[1] have recently called attention to the scantiness of our knowledge concerning the value of the calcium of vegetables for animal nutrition, and have described experiments with dogs in which the calcium was very poorly utilized when derived from carrots.

When that paper was published two of the four experiments reported herein had been completed, but as the results were strikingly different the work was continued with two other subjects. Each of the four subjects had shortly previous to the calcium experiment been the subject of digestion experiments with simple mixed diets, and their digestive capacity was known to be high. The carrot was chosen for special study because it is ingested comparatively easily in large amounts over considerable periods, and because it is a vegetable with a high calcium content.

Methods.

The general plan was to arrange an easily digested diet with a calcium content approximately equal to the estimated minimal requirement for equilibrium in each subject, and to determine calcium balances from the calcium content of analyzed food, urine, and feces. In Series I with two subjects, there were two periods of 3 weeks each. In Period I, milk was made the chief

[1] McClugage, H. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 353.

source of calcium; in Period II, the milk was largely omitted and carrots were introduced to make up the full quota of calcium. It was thought that if the subjects were about in equilibrium on the milk ration, any failure to utilize the calcium would show promptly in the calcium balance. In Series I, the milk period was omitted and the carrot diet consumed for 2 weeks.

Sherman has pointed out[2] that in 63 experiments on ten subjects calcium equilibrium could be maintained on amounts ranging from 0.27 to 0.78 gm. of calcium per 70 kilos of body weight per

TABLE I.

Calcium Content of Foods Used in Diets.

Food material.	Calcium.
	per cent
Bread	0.032
Beef, lean	0.018,* 0.009,† 0.004‡
Milk	0.116
Honey, strained	0.004
Butter	0.014,* 0.010†
Peaches	0.006
Apple	0.010,* 0.0024†
Tomato juice	0.015
Rice	0.012
Coffee infusion	0.003
Carrots	0.044,* 0.052†
Soda crackers	0.025

* Series I.
† " II.
‡ " II, Periods II and III.

day with an average of 0.45 gm. In these experiments the milk diets yielded 0.48 and 0.50 gm. per 70 kilos and the carrot diets from 0.39 to 0.46 gm. Only distilled water was used throughout.

Urine and feces were collected daily and combined into 4 day periods for analysis. The carrots were boiled in their skins, in just enough water to steam them, then the skins removed, the carrots mashed, mixed, and sampled for analysis. Calcium was determined by McCrudden's method.

The calcium content of the foods used in the daily diets is given in Table I.

[2] Sherman, H. C., Chemistry of food and nutritition, New York, 2nd edition, 1918, 264.

EXPERIMENTAL.

In Series I, the two young women had so nearly the same energy requirement that they decided to eat exactly the same amounts of food throughout. The time was divided into two periods of 3 weeks each. In Period I, 70 per cent of the calcium intake came from milk; in Period II, 21 per cent from milk and 55 per cent from carrots. The calcium content of the daily diets is given in Table II.

TABLE II.

Calcium Content of Daily Diets. Series I.
Subjects E. D. B. and R. S. E.

Food.	Period I.		Period II.	
	Weight.	Calcium.	Weight.	Calcium.
	gm.	*gm.*	*gm.*	*gm.*
Bread	152	0.0476	152	0.0477
Meat	100	0.0169	50	0.0086
Milk	226	0.2644	57	0.0668
Honey	31	0.0011	31	0.0015
Butter	50	0.0072	50	0.0072
Sugar	30		50	
Rice	57	0.0069	29	0.0035
Peaches	106	0.0068		
Apple	100	0.0100		
Tomato juice	100	0.0150		
Coffee infusion	200	0.0063	200	0.0057
Carrots			400	0.1744
Total per day		0.3822		0.3154
" " kg. R. S. E		0.0068		0.0056
" " " E. D. B		0.0071		0.0058

The lower calcium intake in Period II was regrettable as it introduced the possibility of an actual calcium deficiency, but the final balances showed that requirement was fully met in the case of E. D. B., though perhaps not quite in that of R. S. E. This change came about through the inability of the subjects to consume as large amounts of the carrots as they had intended. On account of the bulkiness of the diet, the rice was reduced in Period II. The tomato juice, peaches, and apple used in Period I to keep the diet laxative were dispensed with when carrots were introduced.

Both subjects lost calcium during the first 4 day period. This was attributed to their changing from a high to a low calcium

TABLE III.

Daily Intake and Output of Calcium, Series I.

Subject E. D. B., weight 54 kg.

Series.	Period.	Time.	Average daily intake of calcium.	Average daily output of calcium.			Calcium balance.
				Urine.	Feces.	Total.	
		days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
I	I	4	0.383	0.086	0.553	0.639	−0.225
	II	4	0.383	0.081	0.290	0.371	+0.012
	III	4	0.383	0.071	0.243	0.313	+0.070
	IV	4	0.383	0.069	0.215	0.284	+0.099
	V	5	0.383	0.056	0.267	0.323	+0.060
Average.....	II–V	17	0.383	0.070	0.254	0.323	+0.060
II	VI	4	0.315	0.033	0.133	0.166	+0.149
	VII	4	0.315	0.045	0.165	0.210	+0.105
	VIII	4	0.315	0.076	0.236	0.312	+0.004
	IX	4	0.315	0.076	0.235	0.311	+0.003
	X	5	0.315	0.060	0.239	0.299	+0.016
Average.....	VI–X	21	0.315	0.058	0.202	0.260	+0.055
Subject R. S. E., weight 56 kg.							
I	I	4	0.383	0.064	0.567	0.632	−0.249
	II	4	0.383	0.070	0.264	0.334	+0.049
	III	4	0.383	0.069	0.242	0.311	+0.072
	IV	6	0.383	0.069	0.191	0.260	+0.121
Average.....	II–IV	14	0.383	0.069	0.226	0.296	+0.087
II	V	4	0.315	0.074	0.287	0.361	−0.046
	VI	4	0.315	0.077	0.189	0.266	+0.049
	VII	4	0.315	0.073	0.327	0.400	−0.084
	VIII	4	0.315	0.072	0.220	0.292	+0.023
	IX	5	0.315	0.079	0.287	0.366	−0.050
Average.....	V–IX	21	0.315	0.075	0.262	0.336	−0.022

intake and attaining equilibrium on a new level, and the period has been excluded from the averages for the period, though its inclusion would make apparently a better case for the calcium

of carrots. No such loss occurred on going from the milk to the carrot diet. Some loss at this time occurred in the case of R. S. E. who was the most liable to digestive disturbances of all the subjects, and found the carrot diet slightly less satisfactory than the others. The data for the calcium balances are given in Table III.

TABLE IV.

Calcium Content of Daily Diets. Series II.

Subject E. H.

Food.	Period I.		Period II.		Period III.
	Weight.	Calcium.	Weight.	Calcium.	Calcium.
	gm.	gm.	gm.	gm.	gm.
Meat	100	0.0090	150	0.0100	0.0060
Crackers	85	0.0213	100	0.0250	0.0250
Butter	70	0.0070	100	0.0100	0.0100
Sugar	100		100		
Apple	100	0.0024			
Carrots	466	0.2423	400	0.2080	0.2080
Total per day		0.2820		0.2530	0.2490
" " kg.		0.0063		0.0056	0.0055

Subject E. W.

Food.	Period I.		Periods II and III.	
	Weight.	Calcium.	Weight.	Calcium.
	gm.	gm.	gm.	gm.
Meat	100	0.0090	100	0.0040
Soda crackers	150	0.0375	150	0.0375
Sugar	100		100	
Apple	100	0.0024	100	0.0024
Carrots	400	0.2080	533	0.2770
Total per day		0.2569		0.3209
" " kg.		0.0054		0.0067

Digestive disturbances at the beginning and end of Period II (with carrots) in the case of R. S. E. show their effect in the negative calcium balance in both periods. It is worth noting, however, that on this low calcium intake distinct gains were made in three of the five periods and that if all the loss is charged to the carrot calcium the amount retained still represents 88 per cent of the intake.

In Series II, the allowance of calcium was practically the same as in the carrot diets of Series I. For lack of time, the milk period had to be omitted and the two young women who served as subjects subsisted for 12 days on a diet in which carrots furnished from 78 to 86 per cent of the total calcium. The daily diets are given in Table IV.

Slight changes in the diet were made after the first 4 days as E. H. found she could not eat so much carrot and E. W. that she could eat more. E. H. found the carrot diet sufficiently laxative

TABLE V.

Daily Intake and Output of Calcium. Series II.

Period.	Time.	Average daily intake of calcium.	Average daily output of calcium.			Calcium balance.
			Urine.	Feces.	Total.	
Subject E. H., weight 45.5 kg.						
	days	*gm.*	*gm.*	*gm.*	*gm.*	
I	4	0.282	0.058	0.198	0.256	+0.026
II	4	0.253	0.075	0.125	0.200	+0.053
III	4	0.249	0.081	0.217	0.298	−0.049
Average I–III.	12	0.261	0.071	0.180	0.251	+0.010
Subject E. W., weight 48 kg.						
I	4	0.257	0.083	0.166	0.249	+0.008
II	4	0.321	0.062	0.160	0.222	+0.099
III	4	0.321	0.050	0.130	0.180	+0.140
Average I–III.	12	0.300	0.065	0.152	0.217	+0.082

without the apple used for that purpose in Period I. The meat used was somewhat fatter and had a lower calcium content in the later periods. The daily intake and output for each subject are shown in Table V.

SUMMARY.

Two series of experiments to determine the utilization of the calcium of carrots by the human body have been carried out on four healthy young women. The calcium intake was in every case close to the estimated minimum for equilibrium.

Average Daily Intake and Output of Calcium.

Series.	Subject.	Diet.	Average daily intake of calcium.	Calcium.					
				From carrots.	Average daily output.			Balance.	Gain or loss.
					Urine.	Feces.	Total.		
			gm.	*per cent*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
I	E. D. B.	Milk.	0.383		0.070	0.254	0.323	+0.060	+15.6
I	"	Carrot.	0.315	55	0.058	0.202	0.260	+0.055	+17.4
I	R. S. E.	Milk.	0.383		0.069	0.226	0.296	+0.087	+22.7
I	"	Carrot.	0.315	55	0.075	0.262	0.336	−0.023	− 7.3
II	E. H.	"	0.261	84	0.071	0.180	0.251	+0.010	+ 3.8
II	E. W.	"	0.300	86	0.065	0.152	0.217	+0.082	+26.6

In all cases but one there was a positive calcium balance on the carrot diet, and in this case the loss was small. When approximately 55 per cent of the calcium was derived from carrots, one subject had practically the same retention as on a diet in which 70 per cent of the calcium was derived from milk. It seems possible, therefore, to meet the requirement of the adult human organism for calcium largely, if not wholly, from carrots.

EFFECTS OF FEEDING WITH CALCIUM SALTS ON THE CALCIUM CONTENT OF THE BLOOD.

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The advent of micro methods for the determination of blood calcium has opened up many interesting lines of work in a field hitherto practically closed on account of the relatively large amount of blood required for a single analysis, when the older gravimetric procedures are used. Somewhat more than a year ago there was begun in this laboratory a systematic study of the calcium content of the blood of persons suffering from a number of maladies for the relief of which calcium administration has been advocated, and of the effect on the blood calcium produced by the administration of calcium salts to these patients.

As a necessary preliminary to the interpretation of the results obtained on pathological material, we have carried out a number of experiments along the same lines on normal individuals and on animals, the results of which are described in the present paper. Our calcium determinations have been made by Lyman's (1) method, which has been modified in a few minor details.

All determinations have been made on citrated plasma (0.1 gm. of solid sodium citrate to 10 cc. of blood) as preliminary experiments convinced us that no calcium was precipitated by the use of this amount of sodium citrate, and that, as pointed out by Halverson, Mohler, and Bergeim (2), the determination of calcium in plasma or serum is of more significance than when made on whole blood, as originally recommended by Lyman.

In Table I we have collected the results obtained on five men and one woman, all normal subjects, who took by mouth daily three 2 gm. portions of calcium lactate, for a period of 6 to 10 days. During the period of calcium ingestion these subjects were under no regulations as regards food, but continued to eat their ordinary mixed diet.

As will be seen no effect on blood calcium was obtained by the daily ingestion of 6 gm. of calcium lactate in our normal human subjects. Attempts to give larger doses to men proved unsuccessful on account of the disagreeable taste of the various calcium salts tried, so that we were obliged to turn to animals for further experiments on the effects of relatively high calcium dosage.

As experimental subjects we have used cats and rabbits, as representing types of animals that ordinarily eat food differing widely in calcium content. These animals were kept in the

TABLE I.

Experimental Results Obtained on Feeding Calcium Lactate (6 Gm. per Day) to Normal Subjects.

Subject No.	Time.	Calcium per 100 cc. of plasma.
	days	*mg.*
1	1	7.6
	6	7.6
2	1	9.2
	6	9.1
3	1	11.0
	6	10.8
4	1	8.6
	6	9.2
5	1	11.8
	6	11.9
6	1	11.7
	10	11.8

laboratory and fed on the diet to be used during the experiment for 1 week before the initial samples of blood were taken. The food of the rabbits consisted of oats, cabbage, celery tops, and lettuce. The cats were given cooked lean meat (beef and mutton) in unmeasured amounts and in addition each cat received 50 cc. of milk per day.

In Table II we have collected the data obtained on seven cats to which calcium lactate was fed for periods varying from 15 to 38 days. While the number of experimental animals is rather

limited it would seem that it is sometimes possible by feeding with calcium salts to increase the calcium content of the blood of these animals, but that in certain instances calcium administration, even when continued for long periods, is without effect. There is apparently some relation between the initial calcium

TABLE II.

Experimental Results Obtained on Feeding Calcium Lactate to Cats.

Cat No.	Date.	Calcium per 100 cc. of plasma.	Remarks.
	1919	*mg.*	
1	Mar. 2	4.1	Male, weight 3,200 gm.; 0.5 gm. calcium lactate per day.
	" 27	7 3	
	June 11	5.2	2nd experimental period; 1.0 gm. calcium lactate per day.
	" 29	14.2	
2	Apr. 7	6.1	Female, weight 1,820 gm.; 0.5 gm. calcium lactate per day.
	" 27	7.2	
	June 11	7.6	2nd experimental period; 1.0 gm. calcium lactate per day.
	" 29	10.6	
3	Apr. 27	4.8	Male, weight 6,300 gm.; 0.5 gm. calcium lactate per day.
	May 12	5.7	
4	June 19	10.3	Female, weight 1,750 gm.; 0.5 gm. calcium lactate per day.
	" 29	10.4	
	July 11	11.4	2nd experimental period; 1.0 gm. calcium lactate per day.
	" 24	11.8	
5	Sept. 26	10.8	Male, weight 3,000 gm.; 1.0 gm. calcium lactate per day.
	Dec. 2	10.9	
6	Sept. 26	10.4	Male, weight 2,320 gm.; 1 gm. calcium lactate per day.
	Dec. 2	10.3	
7	July 26	11.0	Female, weight 1,950 gm.; 1.0 gm. calcium lactate per day.
	Dec. 2	11.4	

content of the plasma and the ease with which it is possible to effect changes in the blood by experimental means. Thus it will be noted that the calcium content of the plasma of Cats 1, 2, and 3 was rather low in the first sample, and that these animals responded positively to the administration of calcium salts,

whereas the plasma calcium of Cats 5, 6, and 7, for the species of animal, is rather high in initial value, and that calcium feeding was with them without demonstrable effect on the blood.

TABLE III.

Experimental Results Obtained on Feeding Calcium Lactate to Rabbits.

Rabbit No.	Date.	Calcium per 100 cc. of plasma.	Remarks.
	1919	*mg.*	
1	Feb. 16	11.2	Male, weight 2,100 gm.; 0.5 gm. calcium lactate per day.
	" 28	11.0	
18	July 24	13.5	Male, weight 1,800 gm.; 1.0 gm. calcium lactate per day.
	" 29	15.7	
4	Apr. 9	8.3	Female, weight 1,800 gm.; 0.5 gm. calcium lactate per day.
	" 27	8.2	
5	June 2	14.4	Female, weight 1,920 gm.; 1.0 gm. calcium lactate per day.
	" 11	14.2	
17	Sept. 25	14.4	Male, weight 2,240 gm.; 1.0 gm. calcium lactate per day.
	Dec. 1	14.3	
19	Sept. 25	16.0	Male, weight 2,100 gm.; 1.0 gm. calcium lactate per day.
	Dec. 1	15.8	
20	Sept. 25	8.2	Female; 5 young born 1 week before beginning of experiment; lactation continued for 4 weeks.
	Dec. 1	16.0	
21	Sept. 25	14.8	Female, weight 1,660 gm.; 1.0 gm. calcium lactate per day.
	Dec. 1	16.0	
22	Sept. 26	9.1	Female; on Oct. 6th delivered 6 young, which she nursed for about 4 weeks.
	Dec. 2	11.6	

The experimental results obtained on nine rabbits are summarized in Table III. As in the case of the cats but little effect on plasma calcium can be seen except in the case of Rabbits 20 and 22, both of which were lactating during a considerable portion of the experimental period, a fact which may account for the low initial values.

SUMMARY.

The result of a study of the effect of the administration of calcium salts by mouth to men, cats, and rabbits indicates that in most cases it is impossible to increase the concentration of calcium in the plasma by ingestion of calcium salts, but that in cats and rabbits where the initial concentration is low it is sometimes possible to greatly increase the amount of calcium in plasma by feeding salts of this element.

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DETERMINATION OF MAGNESIUM IN BLOOD.

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(Received for publication, January 30, 1920.)

In the course of an investigation of certain problems of calcium metabolism it became desirable to make determinations of magnesium in small amounts of plasma. The method described below has been worked out with this end in view, and has been adapted for use with the filtrate obtained after the precipitation of calcium in plasma or whole blood by Lyman's (1) method.

Briefly stated the procedure consists of the removal of organic material contained in the filtrate from the calcium determination, the precipitation of magnesium as magnesium ammonium phosphate, and the nephelometric determination of the phosphate in this compound by the reagent of Pouget and Chouchak (2). The detailed description of the method follows.

5 cc. of citrated plasma, serum, or whole blood are measured into 15 cc. of 6.5 per cent trichloroacetic acid solution, and after shaking the mixture is allowed to stand for at least 30 minutes, and is then filtered through a dry filter. 10 cc. of this filtrate (equivalent to 2.5 cc. of serum) are used for the determination of calcium according to the method of Lyman. In this method calcium is precipitated essentially according to the well known technique of McCrudden (3) and the crystals of calcium oxalate are then collected and washed by the help of the centrifuge. For the determination of magnesium the supernatant liquid remaining in the centrifuge tubes is collected by means of a small siphon, as is also the portion of ammonium oxalate solution used as wash liquid; the total amount of fluid collected in this way is placed in a flat-bottomed platinum dish and evaporated to dryness after the addition of 3 cc. of 10 per cent sulfuric acid; the residue in the dish is then ignited over a free flame until white, an operation which should be complete in 2 or 3 minutes. When cool the

white residue is dissolved in about 5 cc. of distilled water, and 10 per cent hydrochloric acid is added drop by drop until the solution of ash is acid to methyl orange. This solution is transferred quantitatively to a 100 cc. beaker, using distilled water to conclude the operation, and evaporated to a volume of 2 to 3 cc.; concentrated ammonium hydroxide is then added drop by drop until the solution is alkaline, and finally 0.5 cc. of 10 per cent ammonium phosphate solution containing 50 cc. of concentrated ammonium hydroxide per liter. The beaker is covered with a watch-glass and allowed to stand over night. The next day the liquid is poured into a conical centrifuge tube, and the beaker washed with 20 per cent alcohol containing 50 cc. of concentrated ammonium hydroxide per liter. After centrifuging, the liquid in the tube is removed by means of a small siphon, and the beaker and tube are again washed with about 10 cc. of the alcohol-ammonia mixture. Three more portions of wash liquid should be used, the precipitate being thoroughly stirred after each addition of fresh liquid.

The precipitation and subsequent washing just described are essentially the process described by Marriott and Howland (4) in their micro method for the determination of magnesium in blood.

This portion of the procedure is at once the most important and the most unsatisfactory feature of the determination. Both in the method of Marriott and Howland and in the procedure described in this paper, recourse has of necessity been had to a principle which every chemist will recognize as theoretically incorrect; *viz.*, the determination of magnesium by the measurement of the phosphate combined as ammonium magnesium phosphate, the precipitation of which latter compound must be carried on in the presence of a large excess of phosphate. It is obvious that extremely careful work is essential in the washing of the precipitate in order to avoid contamination with residual traces of ammonium phosphate; furthermore as is well known, it is possible to overwash an ammonium magnesium phosphate precipitate, so that low results are obtained.

After the removal of the last portion of wash liquid, the tube and beaker are allowed to stand, preferably on a water bath or register until the ammonium has evaporated, and the ammonium magnesium phosphate is then dissolved in 10 cc. of 0.1 N hydrochloric acid and transferred to a 100 cc. volumetric flask by means of distilled water. The solution is then made to volume with

distilled water, mixed, and the phosphate determined by means of the strychnine molybdate reagent.[1]

For the determination of magnesium in normal plasma or serum 25 cc. of the above solution are usually the most convenient to use, but larger or smaller amounts are sometimes called for.

To the amount of solution taken is added a quantity of distilled water sufficient to bring the volume to 50 cc., then 25 cc. of the strychnine molybdate reagent are added. After standing for a period of 5 minutes the suspension is read against a standard containing 0.01 mg. of magnesium in a volume of 50 cc.[2] to which have been added 25 cc. of the strychnine molybdate reagent, and which has been allowed to stand for the same length of time as the unknown.

The procedure as outlined above has been carried out in pure solutions containing 0.02 to 0.10 mg. of magnesium, and on similar amounts of magnesium added to serum and plasma. The average recovery in these experiments has been 94 per cent.

The magnesium in more than 100 determinations made on human blood serum (many of which were on pathological cases) has given figures varying from 0.8 to 3.8 mg. per 100 cc. of plasma. In the more strictly normal material, values from 1.6 to 3.5 mg. have been obtained.

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[1] For this reagent the modification described by Bloor (5) gives most satisfactory results.

[2] This standard is prepared from ammonium magnesium phosphate by dissolving 1.02 gm. of the pure salt in 100 cc. of N hydrochloric acid and diluting to a volume of 1 liter with distilled water; 1 cc. of this solution is equivalent to 0.10 mg. of magnesium. For the preparation of this dilute standard a portion of the strong solution is diluted 50 times with 0.1 N hydrochloric acid so that 5 cc. of the resulting solution will be equivalent to 0.01 mg. of magnesium.

A SYSTEM OF BLOOD ANALYSIS.

SUPPLEMENT I.

A SIMPLIFIED AND IMPROVED METHOD FOR DETERMINATION OF SUGAR.

BY OTTO FOLIN AND HSIEN WU.

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(Received for publication, January 26, 1920.)

In our system of blood analysis published a short time ago[1] we described a new method for the determination of sugar. In this method the sugar is oxidized by a weakly alkaline copper tartrate solution and the cuprous copper formed is estimated colorimetrically by the help of the phenol reagent of Folin and Denis. In this determination the errors due to creatine, creatinine, and uric acid are eliminated while a new source of error, namely that due to the so called phenols, is introduced. This last named error is certainly much smaller than the errors due to creatine, creatinine, and uric acid in other methods, so that in point of accuracy our method should be fully as good as any other known method. We could have eliminated any error due to the so called phenols by simply omitting the addition of sodium carbonate, because the phenol reagent reacts with cuprous copper in acid solution. Incidentally we should thus also eliminate the blank due to the alkaline copper tartrate. The process so obtained seemed to us less satisfactory, however, for the reason that the phenol reagent has a pronounced yellow color.[2] The matching of the colors in the colorimeter is rendered difficult in consequence and uncertain under such conditions, and would not [illegible] when the standard and the unknown are [illegible] can Association [illegible] the [illegible] edict [illegible] ned ou[illegible]

[1] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 106.

[2] The phenol reagent sometimes has a greenish color due to some reduction by organic dusts.

strength. We accordingly chose to destroy the surplus phenol reagent by the addition of sodium carbonate.

In the course of subsequent studies of the method we have found a process by which the disturbing effects of the phenols can be eliminated. To accomplish this purpose we have replaced the regular phenol reagent of Folin and Denis by a reagent which reacts with cuprous copper, in acid solution, yet gives no color with phenols. This new reagent is prepared as follows:

Transfer to a liter beaker 35 gm. of molybdic acid and 5 gm. of sodium tungstate. Add 200 cc. of 10 per cent sodium hydroxide and 200 cc. of water. Boil vigorously for 20 to 40 minutes so as to remove nearly the whole of the ammonia present in the molybdic acid.[3] Cool, dilute to about 350 cc., and add 125 cc. of concentrated (85 per cent) phosphoric acid. Dilute to 500 cc.

It will be seen that the preparation of this reagent is much simpler than the preparation of the phenol reagent. The solution has none of the yellow color of the phenol reagent, yet gives an intense blue color with cuprous oxide. Since the reaction takes place in acid solution the blue color of the alkaline copper tartrate is also eliminated. The sodium tungstate contained in this reagent is added because there is sodium tungstate in our blood filtrates, and tungstates modify somewhat the shade of blue obtained in the reaction.

The alkaline copper solution has not been changed. Dissolve 40 gm. of pure anhydrous sodium carbonate in about 400 cc. of water and transfer to a liter flask. Add 7.5 gm. of tartaric acid, and when the latter has dissolved add 4.5 gm. of crystallized copper sulfate. Mix and make up to a volume of 1 liter. If the chemicals used are not pure a sediment of cuprous oxide may form in the course of 1 or 2 weeks. If this should happen, remove the clear supernatant reagent with a siphon, or filter through a good quality filter paper. Our reagent seems to keep indefi[illegible] To test for the absence of cuprous copper in the sol[illegible] 2 cc. to a test-tube and add 2 cc. of the molyb[illegible]ution; the deep blue color of the copper should [illegible] com[illegible] anish. In order to forestall improper use of [illegible]agent attention should be called to the fact that it contains

[3] The molybdic acid we used was obtained from the Primos Company, Primos, Pa., and it contained considerable ammonia.

extremely little alkali, 2 cc. by titration (using the fading of the blue copper tartrate color as indicator), requiring only about 1.4 cc. of normal acid.

Standard Sugar Solutions.—Three standard sugar solutions should be on hand: (1) a stock solution, 1 per cent dextrose or invert sugar, preserved with xylene or toluene; (2) a solution containing 1 mg. of sugar per 10 cc. (5 cc. of the stock solution diluted to 500 cc.); (3) a solution containing 2 mg. of sugar per 10 cc. (5 cc. of the stock solution diluted to 250 cc.). The invert sugar solution has the advantage that it can be easily prepared from cane sugar, which is pure. The keeping quality of such solutions should be less good than those made from glucose, but we have encountered no trouble on that score. When good quality glucose is available, it is, of course, the one to use. The diluted solutions should be preserved with a little added toluene or xylene; it is probably better not to depend on such diluted solutions to keep for more than a month, but the stock solution should keep indefinitely.

It is a well known fact that the cuprous compounds produced by sugar in alkaline copper solutions show a marked tendency to be reoxidized to the cupric condition when exposed to air. Most of us are all too familiar with that fact in connection with ordinary sugar titrations done by overcautious students. That such reoxidation must occur to some extent in our colorimetric blood sugar determination is also undeniable. Without having made any direct experiments on the extent of such reoxidations we had satisfied ourselves that they do not contribute any material error in our sugar determinations. Check experiments with sugar solutions 50 per cent apart and heated 4, 6, and 8 minutes had given proportionate values. And in actual blood sugar determinations the values obtained were not changed by varying the heating time from 4 to 8 minutes. We had therefore no occasion to fear that material analytical errors could creep in because of the reoxidation.

At the last annual meeting of the American Association of Biological Chemists (Cincinnati, 1919), Benedict condemned our blood sugar method on the ground of excessive, inevitable, and uncontrollable reoxidations of cuprous oxide. He cited shaking experiments by means of which more than 60 per cent were made

to disappear. He also asserted that reoxidations are much more extensive in blood filtrates than in pure sugar solutions and therefore insisted that the blood sugar values obtained by our method must be too low. Vigorous shaking, as it happens, is also disastrous to the reaction between reducing sugar and alkaline picrates. Losses of 40 per cent can be secured by shaking (in 25 cc. flasks), and if agitation by an air current is substituted for shaking, nearly the whole of the sugar represented in a blood sugar determination by Benedict's method is lost. We cite these observations not as a criticism of Benedict's method but merely to show the grossly misleading character of shaking experiments.

It must be admitted nevertheless that we made something of an error in depending exclusively on indirect evidence on so important a point as the losses of cuprous oxide, and we gladly give Benedict credit for having compelled us to reexamine our method with reference to the effect of reoxidation. We have verified our earlier findings that analytical errors do not occur because of such reoxidation, but this is because all our test-tubes in which the oxidation of sugar takes place are of substantially the same diameter, 17 to 18 mm. on the inside. The oxidation in such tubes, when kept in a somewhat slanting position, may amount to as much as 20 per cent of the cuprous oxide formed, yet correct sugar values are obtained.

In order to get data on the reoxidation problem we have conducted the reduction in test-tubes of different internal diameters ranging from 4 to 23 mm. With test-tubes in a vertical position there is practically no loss of cuprous oxide by reoxidation until the diameter of the tube is at least 15 mm. At about 20 mm. or above the losses become astoundingly large. Currents having a somewhat similar effect as shaking must come into play in such tubes for the losses are not at all proportionate to the areas of the surface of solution. Shaking was of course excluded. The results of two series of experiments of the kind indicated are shown in Table I.

The remarkable fact about these reoxidations is that the percentage loss of copper so produced is not materially dependent on the amount of cuprous oxide involved. In a given tube the percentage loss is substantially the same whether one works with 0.1 or 0.4 mg. of sugar, whether with pure sugar solution or our blood

filtrate, and this is manifestly the reason why our original method has given uniformly concordant values. The results recorded in Table II show that the per cent loss of copper is almost independent of the amount of reduction involved. The reductions were made simultaneously in test-tubes of definite but widely different internal diameters.

TABLE I.

Relation Between Area of Surface of Solution and Loss of Cuprous Oxide by Reoxidation, Using 0.1 Mg. of Sugar.

Internal diameter of tube at level of surface of solution.	Colorimeter reading.	Loss.
mm.		*per cent*
4*	19.8	0
6	20	0
9	20	0 (?)
11	20	0 (?)
14	20, 20.5	0, 3
16	21.4, 22.5	7, 13
19	27.5, 29.5	27, 32
23	31, 32	36, 36

* In hydrogen atmosphere.

TABLE II.

Showing that Percentage Loss of Cuprous Oxide by Reoxidation Is not Materially Affected by Amount of Sugar Used for Reduction.

Amount of sugar used.	Colorimeter readings.		
	Constricted tube, 4 mm.	Open tube, 16 mm.	Open tube, 20 mm.
mg.			
0.1	20	22	28
0.2	20	21.8	
0.4	20	22	26
0.8	10	11	

In view of these findings it is necessary to be more precise than we had been in our description of the kind of test-tubes to be used. We prescribed test-tubes having the dimensions 200 × 20 mm. because ours were bought under those specifications, whereas in point of fact their internal diameter is only 17 to 18 mm. The subject is of sufficient importance to call for a special

tube by the use of which reoxidation is automatically excluded. This special blood sugar test-tube is shown in Fig. 1. The essential point to be observed in connection with it is, of course, that the surface of the alkaline mixture of sugar and copper shall reach the constricted part.

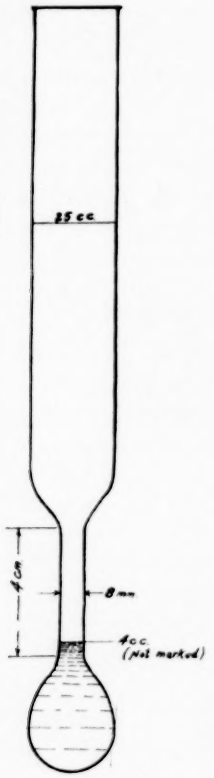

FIG. 1. Blood sugar tube.

Test-tubes of this sort, with and without graduation, are now made by The Emil Greiner Company, 55 Fulton Street, New York, and can also be obtained from Arthur H. Thomas Company, West Washington Square, Philadelphia. For the benefit of those who wish to make and graduate their own test-tubes we

suggest that a stem of about the size of a cherry stem should first be attached to the bottom of the test-tube. This stem is best produced by sealing on a piece of a broken test-tube and then drawing it out. If a good blast lamp is available, the whole process of making a tube does not take more than 2 or 3 minutes.

The blood sugar determination is now made as follows:

Transfer 2 cc. of the tungstic acid blood filtrate to a blood sugar test-tube, and to two other similar test-tubes (graduated at 25 cc.) add 2 cc. of standard sugar solution containing respectively 0.2 and 0.4 mg. of dextrose. To each tube add 2 cc. of the alkaline copper solution. The surface of the mixtures must now have reached the constricted part of the tube. If the bulb of the tube is too large for the volume (4 cc.) a little, but not more than 0.5 cc., of a diluted (1 : 1) alkaline copper solution may be added. If this does not suffice to bring the contents to the narrow part, the tube should be discarded. Test-tubes having so small a capacity that 4 cc. fills them above the neck should also be discarded. Transfer the tubes to a boiling water bath and heat for 6 minutes. Then transfer them to a cold water bath and let cool, without shaking, for 2 to 3 minutes. Add to each test-tube 2 cc. of the molybdate phosphate solution. The cuprous oxide dissolves rather slowly if the amount is large but the whole, up to the amount given by 0.8 mg. of dextrose, dissolves usually within 2 minutes. When the cuprous oxide is dissolved, dilute the resulting blue solutions to the 25 cc. mark, insert a rubber stopper, and mix. It is essential that adequate attention be given to this mixing because the greater part of the blue color is formed in the bulb of the tube.

The two standards given representing 0.2 and 0.4 mg. of glucose are adequate for practically all cases. They cover the range from about 70 to nearly 400 mg. of glucose per 100 cc. of blood.

It will be noted that in the process described we prescribe cooling of the alkaline cuprous oxide suspension before adding the phosphate molybdate solution. This cooling is not essential and, in case of one or two determinations only, may be omitted. In a large series of determinations it is probably best to use it. The important point is that the standard and the unknowns should not only be heated the same length of time but should also have

substantially the same temperature when the acid reagent is added. The maximum color develops faster in hot solutions; but if a reasonable uniformity of condition is maintained it makes no difference whether the color comparison is made at the end of 5 minutes or at the end of 1 hour.

TABLE III.

Comparison of Blood Sugar Values per 100 Cc. Obtained by Original and Revised Methods.

Source.	Revised method.	Original method.	
		Open tube.	Constricted tube.
	mg.	mg.	mg.
1* Human.	30	34	
2 "	61	65	
3 "	90	91	
4 "	99	93	
5 "	105	108	
6 "	90	98	
7 "	105	121	
8 "	121	129	125
9 "	134	130	130
10 "	131	131	131
11 "	200	210	218
12 "	224	218	228
13 Dog.	86	85	
14 "	84	85	

* Blood 24 hours old.

In our blood sugar method as we now have it reoxidations of the cuprous compounds are excluded; the blank due to the blue alkaline copper tartrate is eliminated, and, finally, the error due to the so called phenols in blood filtrates is removed. From a theoretical standpoint the method now appears to be without a flaw, and from the experimental standpoint it is rather more simple, inasmuch as the addition of sodium carbonate is omitted. The analytical figures in Table III show that the new process tends to give slightly lower values than the original method.

DISTRIBUTION OF THE BASIC NITROGEN IN PHASEOLIN.

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(Received for publication, January 14, 1920.)

Determination of the hydrolytic products of phaseolin, the chief protein of the navy bean, *Phaseolus vulgaris*, has been made by Osborne and Clapp (1). They did not find the amino-acid content essentially different from other vegetable globulins. It contained a sufficient quantity of the amino-acids known to be necessary for normal growth, with the possible exception of cystine. Nutrition experiments, however, by Osborne and Mendel (2), and McCollum, Simmonds, and Pitz (3) with the proteins of this bean met with little success.

TABLE I.

Basic Amino-Acids in Phaseolin.

Amino-acid.	Van Slyke method.	Absolute method, Osborne and Clapp (1).
	per cent	*per cent*
Cystine	0.84	Not determined.
Arginine	6.11	4.87
Histidine	3.32	2.62
Lysine	7.88	4.58

To obtain more information for use in our nutrition experiments with the navy bean, an analysis of phaseolin by the Van Slyke (4) method was made. It will be noted that the results found by this method for the basic amino-acids were higher than those obtained by the direct method of Kossel and Patten (5), the value obtained for lysine being considerably greater. Sulfur determinations on the basic amino-acid fraction indicated the presence of 0.84 per cent of cystine. The accuracy of this method for the determination of cystine is uncertain particularly in cases where the per-

centage of sulfur in proteins is low, since a comparatively large correction has to be applied for the solubility of cystine phosphotungstate. The results of our analysis together with those obtained by Osborne and Clapp are summarized in Table I.

EXPERIMENTAL.

Analysis of Phaseolin by the Van Slyke Method.—Duplicate 3 gm. samples of phaseolin were used, each equivalent to 2.8476 gm. of moisture and ash-free protein and containing 15.99 per

TABLE II.

Analysis of Phaseolin by Van Slyke Method. Nitrogen Corrected for Solubility of Bases.

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0496	0.0495	10.90	10.88	10.89
Humin N adsorbed by lime	0.0079	0.0077	1.73	1.70	1.72
" N in amyl alcohol extract	0.0008	0.0020	0.17	0.45	0.31
Cystine N	0.0027	0.0029	0.59	0.64	0.61
Arginine N	0.0545	0.0576	11.97	12.65	12.31
Histidine N	0.0260	0.0252	5.71	5.53	5.62
Lysine N	0.0427	0.0437	9.38	9.60	9.49
Amino N of filtrate	0.2470	0.2533	54.25	55.63	54.94
Non-amino N of filtrate	0.0209	0.0146	4.59	3.21	3.90
Total N regained	0.4521	0.4565	99.29	100.29	99.79

TABLE III.

Basic Amino-Acids in Phaseolin.

Amino-acid.	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	0.81	0.87	0.84
Arginine	5.94	6.28	6.11
Histidine	3.37	3.27	3.32
Lysine	7.79	7.97	7.88

cent or 0.4553 gm. of nitrogen. Each sample was hydrolyzed for 24 hours by boiling with 100 cc. of 20 per cent hydrochloric acid. The phosphotungstates of the bases were decomposed by the

amyl alcohol-ether method (6). The results of these analyses are recorded in Tables II and III.

SUMMARY.

1. The basic amino-acids of phaseolin were determined by the Van Slyke method of analysis.

2. The percentage of lysine was found to be considerably higher than that obtained by the direct method of Kossel and Patten.

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STUDIES IN NUTRITION.*

II. THE RÔLE OF CYSTINE IN NUTRITION AS EXEMPLIFIED BY NUTRITION EXPERIMENTS WITH THE PROTEINS OF THE NAVY BEAN, PHASEOLUS VULGARIS.

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A great advance was made in our knowledge of the principles of nutrition when it was shown that the nutritive value of proteins depends on the relative proportions of certain amino-acids in the protein molecule. Numerous experiments have demonstrated that the withdrawal or addition of certain amino-acids renders diets deficient or makes them complete for normal growth. It is well known that proteins which lack either lysine or tryptophane fail to promote the growth of animals. It has also been established that certain other amino-acids are necessary for maintenance and growth and that some of the amino-acids usually found in proteins are not essential.

Thus, Ackroyd and Hopkins (1) have shown that arginine and histidine are to some extent interchangeable. Totani (2) does not regard tyrosine as an essential amino-acid and suggests the possibility that phenylalanine may take its place in nutrition. Hopkins (3) has also shown that when both glutaminic and aspartic acids were removed from the products of hydrolysis of a biologically complete protein the resulting amino-acid residue was still adequate for normal growth. The theory that the nutritive value of a protein depends upon its amino-acid content encountered an apparent contradiction in the fact that phaseolin from the navy bean failed to promote growth, although it had been shown by Osborne and Clapp (4) that this protein contains all the known essential amino-acids.

* Read before The National Academy of Sciences, New Haven, 1919.

The nutritive value of phaseolin has been studied by Osborne and Mendel (5) who used the isolated protein in their experiments. The animals failed to grow and died in a relatively short time. McCollum, Simmonds, and Pitz (6) fed navy bean meal as the sole source of protein in the diet and their experiments also resulted in a failure to obtain growth. These authors attribute the failure of the navy bean to promote growth to the presence of hemicelluloses.

In our experiments where navy bean meal was used as the sole source of protein we could not detect any symptoms of tympanites although the animals failed to grow.

In making a study of the chemical analysis of phaseolin published by Osborne and Clapp (4) it seemed that all the known essential amino-acids were present in sufficient quantities to promote growth with the possible exception of cystine. An analysis of phaseolin (7) made by Van Slyke's method did not throw any further light on the subject and gave higher results for the basic amino-acids than those reported by Osborne and Clapp. This still left the question of a possible deficiency of cystine. It was evident that the percentage of cystine in phaseolin cannot be very high since this protein contains but approximately 0.3 per cent of sulfur. If all this belonged to cystine there would be only about 1.2 per cent of this amino-acid in phaseolin. It is probable, however, that only about one-third of the total sulfur in phaseolin represents cystine, as shown by Osborne (8) who determined the percentage of total sulfur liberated when a number of different proteins were boiled with potassium hydroxide. It was assumed that approximately two-thirds of the cystine sulfur of proteins was thus liberated as hydrogen sulfide. This assumption was based on experiments with pure cystine from which approximately two-thirds of the total sulfur was liberated as hydrogen sulfide when treated in the same manner. It is not known in what form the non-cystine sulfur exists in proteins.

In view of the above facts it appeared that the failure of phaseolin to promote growth might be due to a deficiency in cystine. 2 per cent of cystine, prepared from wool, was therefore added to the phaseolin and 18 to 20 per cent of this mixture was used in a diet together with the essential non-protein ingredients. The animals placed on this diet maintained their weight for a con-

siderable time and grew slightly but the results were far from satisfactory. While the addition of cystine had improved the nutritive value of phaseolin the failure to obtain normal growth was still an unsolved problem.

The question of the digestibility of phaseolin was next considered, since metabolism experiments (9, 10) had shown that the nitrogen of the navy bean was not well assimilated. Phaseolin was therefore subjected to digestion with trypsin *in vitro*. The usual procedure was followed, the mixture being made slightly alkaline with sodium carbonate and the digestion carried out at 37°C. To prevent bacterial growth, the phaseolin was heated at 100°C. in air and 0.1 per cent of sodium benzoate was added to the distilled water used in the digestion. When incubation was complete the sodium carbonate was neutralized with hydrochloric acid and the solution evaporated to dryness on a steam bath. The residue was ground to a powder and 2 per cent of cystine was added. When this digested phaseolin, supplemented with cystine, was used as the sole source of protein in the diet, the rats grew normally. There were then several factors to consider. Was the success obtained due to the digestion with trypsin, or the alkaline action of the sodium carbonate, or the heating during the evaporation? It seemed improbable that the sodium benzoate was one of the factors, other than aiding in maintaining sterility. Bacterial counts[1] were made before and after incubation by making cultures on agar. The bacterial content was so low that fermentation from this source was eliminated as a factor. Experiments were made in which the trypsin, sodium carbonate, and sodium benzoate were eliminated one by one, and normal growth was still obtained when the phaseolin was simply suspended in water, boiled for 30 minutes, and the mixture evaporated to dryness, 2 per cent of cystine being added to the residue before preparing the diet. A ration containing phaseolin which had been heated with water, but to which no cystine had been added, was also tried and slow growth was obtained. When cystine was added to this ration the rate of growth became normal. The success of the experiments, therefore, depended on two factors. *Phaseolin is rendered a more efficient food by heating it with water. It must also be supplemented by cystine to obtain normal growth.*

[1] The bacterial counts were made by Miss R. B. Edmondson of the Microbiological Laboratory, Bureau of Chemistry.

It is difficult to explain why heating phaseolin with water renders it more nutritious. This process may cause some molecular rearrangement to occur in the very complex and probably labile protein molecule, thereby making it more readily digestible or the phaseolin may possess a slight toxicity which is destroyed by heating with water.

It now seemed important to ascertain whether or not navy bean meal could be rendered efficient for normal growth by the process mentioned above. The cooked meal had been tried in our earlier experiments but while the rats maintained their weight for a considerable time only slight growth was obtained. The bean meal yielded to treatment in the same manner as phaseolin. When cooked, dried, and supplemented with cystine, it produced normal growth when used as the sole source of protein in the diet.

The importance of cystine as an ingredient of a complete diet has been shown by a number of investigators. Osborne and Mendel (11) have shown that, when casein is supplemented with cystine, 9 per cent of the casein is as effective as 15 per cent of casein alone. This percentage they found to be necessary for normal growth in diets of high calorific value.

Abderhalden (12) conducted nutrition experiments with hydrolyzed proteins from which he had attempted to remove the cystine. He did not reach any definite conclusion, although he states that cystine is apparently necessary. Hopkins and his coworkers (1, 2, 3), in their nutrition experiments with hydrolyzed proteins, always added cystine, since this amino-acid is partly decomposed by heating with acids during the process of hydrolysis. Mitchell (13) and Geiling (14) also added cystine to the diets used in their experiments with white mice when feeding amino-acid mixtures. Lewis (15), experimenting on dogs, showed that if small quantities of cystine were added to diets low in protein the nitrogen elimination of the animals diminished, whereas an equivalent quantity of nitrogen in the form of tyrosine or glycine added to the diet did not diminish the quantity of nitrogen eliminated. It is evident that Lewis was experimenting with diets in which cystine was a limiting factor. Daniels and Rich (16) attempted to substitute inorganic sulfates for cystine but without success.

Casein contains 0.8 per cent of sulfur but Osborne (8) has shown that approximately only 0.15 per cent of the casein is cystine sul-

fur. McCollum, Simmonds, and Pitz (6) found that casein was not a very effective supplement to navy bean proteins. Their observation is now readily interpreted when it is considered that they were supplementing the navy bean protein, which is low in cystine, with casein which also is low in cystine. It seems probable that cystine was the limiting factor in this mixture of proteins. These facts lend a new interest to the experiments of Osborne (8) in which he attempted to ascertain the proportion of cystine sulfur to the total sulfur in a number of proteins. The proteins were boiled with potassium hydroxide and the sulfur liberated as hydrogen sulfide was multiplied by three-halves to obtain the quantity of cystine sulfur in the proteins. The results indicated that the sulfur in the form of cystine varied from approximately 20 to 75 per cent of the total sulfur in the fifteen vegetable and animal proteins examined (17). He noted that casein and phaseolin, which become more efficient when supplemented with cystine, both yield but small quantities of hydrogen sulfide when boiled with alkali. On the other hand such proteins as ovalbumin, fibrin, ovovitellin, and edestin, which are known to be efficient proteins, yield a relatively large quantity of hydrogen sulfide.

The work herein described was reported in a preliminary paper read before the Society of Biological Chemists at the Baltimore meeting in April, 1919. At that time Osborne and Mendel informed us that they had conducted further experiments on the nutritive value of phaseolin but the results of this work had not been published. They also found that the nutritive value of phaseolin was increased by cooking. Furthermore, they found that the protein extracted from the navy bean by sodium hydroxide and reprecipitated by neutralization was more effective than phaseolin prepared by extraction with sodium chloride and precipitated by dialysis. Osborne and Mendel very kindly placed the results of their unpublished work at our disposal and some of the curves shown in this paper are taken from their experiments.

Experiments with Raw and Cooked Phaseolin.—This work was done by Osborne and Mendel who found that when raw phaseolin was the sole source of protein in an otherwise adequate diet the rats declined in weight rapidly and some of them died. When the phaseolin was replaced by casein the growth became normal. By alternating phaseolin with casein in the diet the rats could be

made to lose weight or grow normally. Phaseolin which had been cooked for a short time in water served to maintain weight but produced practically no growth. In all these experiments the water-soluble vitamine and salts were supplied by protein-free milk and the fat-soluble vitamine by butter fat. The curves are shown in Chart 1.

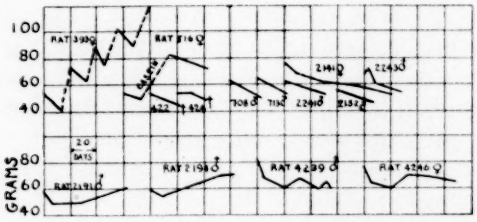

CHART 1. The upper set of curves shows the failure of raw phaseolin to maintain the weight of white rats. In the curves for Rat 393 ♀ and Rat 516 ♀ the solid lines of the curves show the effects of a diet in which phaseolin was the sole source of protein. The broken portions of the curves show the effect of replacing the phaseolin with casein. The daggers indicate that the rats died.

The lower set of curves shows the effect of cooking the phaseolin before preparing the diet. It will be seen that the rats were almost able to maintain their weights for a considerable time but could not grow. The phaseolin was stirred into distilled water and cooked for 5 minutes after the water had been heated to the boiling point. It was then filtered off and dried. The curves in this chart represent the results of unpublished experiments by Osborne and Mendel. The composition of the diets follows.

	Uncooked. gm.	Cooked. gm.
Phaseolin (or casein)	18	18
Protein-free milk	28	28
Starch	28	28
Butter fat		18
Lard	26	8
	100	100

Experiments with Phaseolin Supplemented with Cystine.—The raw phaseolin[2] used in these experiments was supplemented with 2

[2] The phaseolin and cystine used in these experiments were prepared by Mr. C. E. F. Gersdorff of the Protein Investigation Laboratory, Bureau of Chemistry.

per cent of cystine and this mixture was used as the sole source of protein in an otherwise adequate diet. The addition of cystine to the raw phaseolin enabled the rats to grow slowly. A mixture of cooked phaseolin supplemented with cystine at once improved the growth. The replacement of the raw phaseolin supplemented with cystine by casein after the rats had grown but very little for some time also caused a marked improvement in the rate of growth. The results of these experiments are shown in Chart 2.

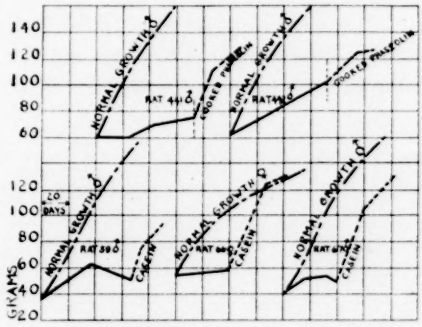

CHART 2. The upper set of curves shows that raw phaseolin supplemented with cystine enabled the rats to grow slowly. During the period indicated by the dotted portion of these curves the rats received cooked phaseolin supplemented with cystine. A marked improvement in the rate of growth occurred at once when the raw phaseolin was replaced by the cooked protein.

In the experiments represented by the lower set of curves the rats received raw phaseolin supplemented with cystine. During the second period, shown by the broken lines, the animals received casein instead of the phaseolin and cystine.

The composition of the diet follows.

	gm.
Phaseolin	17.64
Cystine	0.36
Protein-free milk	28.00
Starch	21.00
Agar	5.00
Butter fat	18.00
Lard	10.00
	100.00

Experiments with Cooked Phaseolin Supplemented with Cystine.—A number of experiments were made with phaseolin cooked under various conditions and supplemented with cystine. In some cases the phaseolin was predigested with trypsin and cooked before adding cystine. Some of the diets contained sodium benzoate which had been used to keep the solution sterile during digestion with trypsin. In all cases the rats grew at a normal rate when the phaseolin was cooked, whether or not it had been predigested, if finally supplemented with cystine. In two experiments the animals received cooked phaseolin for some time without the addition of cystine. The rate of growth was far below normal but became normal when cystine was added to the diet. The results of these experiments are shown in Chart 3.

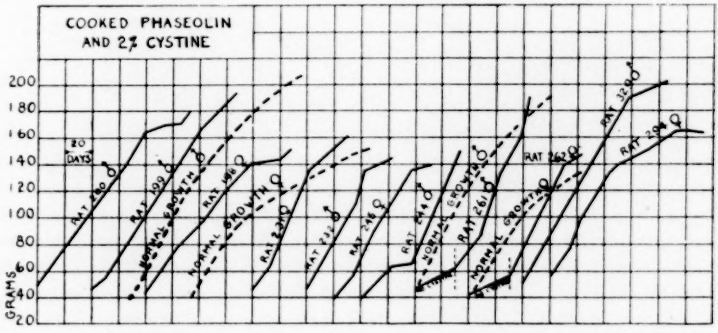

CHART 3. The curves in this chart show that normal growth was obtained on diets containing cooked phaseolin supplemented with cystine. The protein given to Rats 198 ♀, 199 ♂, and 200 ♂ was treated by suspending 12.5 gm. of phaseolin in distilled water to each 100 cc. of which had been added 0.5 gm. of sodium carbonate, 0.1 gm. of sodium benzoate, and 0.05 gm. of trypsin. This mixture was incubated for 5 hours at 37°C., heated to boiling, neutralized with hydrochloric acid, and evaporated to dryness on a steam bath. The residue was ground to a powder and mixed with 2 per cent of cystine. The phaseolin fed to Rats 231 ♀ and 232 ♂ was treated in the same manner but the trypsin was omitted. The phaseolin given to Rats 244 ♂ and 245 ♀ received similar treatment but without the addition of sodium carbonate or trypsin. The neutralization with hydrochloric acid was also omitted. The phaseolin fed to Rats 329 ♂ and 294 ♀ was simply boiled in distilled water for 30 minutes. The mixture was then evaporated to dryness on a steam bath. The residue was ground to a powder and supplemented with 2 per cent of cystine. Rats 261 ♂ and 262 ♀

during the first stage were placed on a diet containing phaseolin cooked in water. The second stage shows the remarkable improvement in the rate of growth due to the addition of 2 per cent of cystine to the cooked phaseolin.

The composition of the diet modified as stated above follows.

	gm.
Phaseolin (cooked, etc.)	19.60
Cystine	0.40
Protein-free milk	28.00
Starch	22.00
Agar	5.00
Butter fat	18.00
Lard	7.00
	100.00

Experiments with Navy Bean Meal.—Osborne and Mendel found that rats fed on raw bean meal declined rapidly in weight and soon died. When the bean meal was cooked slight growth was ob-

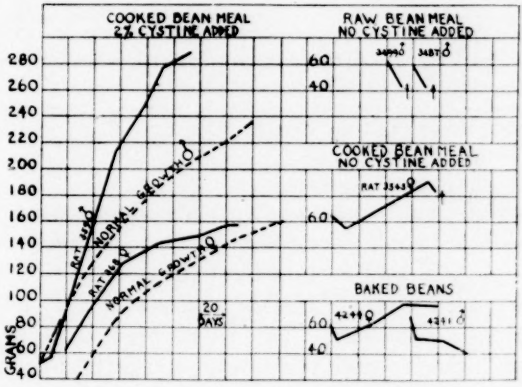

CHART 4. Rats 359 ♂ and 368 ♀ were fed on a diet containing cooked navy bean meal supplemented with cystine equivalent to 2 per cent of the protein. The bean meal was cooked with distilled water for 3 hours. The mixture was then evaporated to dryness and the residue ground to a powder to which cystine was added before preparing the diet. The excellent growth obtained shows that cooked navy beans, supplemented with cystine, furnished an adequate protein mixture.

The following data are taken from results obtained by Osborne and Mendel.

Rats 3499 ♂ and 3487 ♂ were fed on a diet in which the proteins were derived from raw navy bean meal. The curves show clearly the inadequacy

of this diet. Rat 3545 ♀ was fed on a diet in which the protein was furnished by cooked bean meal. It is evident that cooking the bean meal causes an improvement comparable to that resulting from cooking phaseolin. Rats 4244 ♀ and 4241 ♂ were fed on a diet in which the protein was supplied by beans baked without the addition of pork. It is evident that the proteins of the navy bean must be cooked and supplemented with cystine before they can become adequate for normal growth.

The composition of these diets follows.

	Rats 359♂, 368♀.	Rats 3499♂, 3487♂.	Rats 4244♀, 4241♂	Rat 3545♀.
	gm.	gm.	gm.	gm.
Cooked navy bean meal	71.64			
Raw navy bean meal		35		54
Baked beans			72	
Corn gluten		19		
Cystine	0.36			
Salt Mixture IV*	4.00		3	
Protein-free milk		21		22
Butter fat	15.00	18	18	18
Lard	9.00	7	7	6
	100.00	100	100	100

* For composition of salt mixture see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 374.

tained for some time. Baked beans showed no greater efficiency than cooked bean meal. We found that cooked navy bean meal supplemented with cystine equivalent to 2 per cent of its protein produced normal growth. This diet contained butter fat to furnish fat-soluble vitamines. The salts were furnished by an artificial salt mixture and the beans supplied sufficient water-soluble vitamine. The curves are shown in Chart 4.

Similar experiments are being made on the proteins of other leguminous seeds and the results obtained will be reported in future publications.

SUMMARY.

1. Cystine has been shown to be essential for normal growth.

2. Phaseolin, the principal protein of the navy bean, is rendered a more efficient food by heating with water.

3. Cooked phaseolin or cooked navy bean meal when supplemented with cystine furnished adequate protein for normal growth.

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STUDIES IN NUTRITION.

III. THE NUTRITIVE VALUE OF COMMERCIAL CORN GLUTEN MEAL.

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The fact that corn, *Zea mays*, is the most abundant cereal grown in the United States has led a number of investigators to study the nutritive value of its proteins. Among such studies are those made by Osborne and Mendel (1, 2, 3, 4), McCollum, Simmonds, and Pitz (5), Hart, Halpin, and McCollum (6), and Hogan (7, 8). These researches have contributed greatly to our present knowledge of the nutritive value of corn proteins.

Corn is known to contain several different proteins (9, 10) and the approximate distribution of these in the kernels of a sample of corn containing a relatively high percentage of proteins is shown in the following table which is taken from a publication by Osborne and Mendel (2).

	Per cent of protein.
Globulins + albumins + "proteoses"	21.9
Zein	41.4
Maize glutelin	30.8
Insoluble in alkali	5.9
	100.0

Zein, which constitutes approximately from 41 to 52 per cent of the proteins in corn, lacks lysine and tryptophane, which are essential amino-acids for the growth of animals. It also contains but small quantities of the basic amino-acids which are also essential in a complete diet. Corn glutelin, which is the next most abundant protein, contains all the essential amino-acids which zein lacks and promotes normal growth. Osborne and Mendel

(11) also state that considerable growth can be obtained from corn embryos as the sole source of protein, likewise with a mixture of corn gluten and corn embryo when a sufficient quantity of the latter is present. We have, therefore, in corn a mixture of efficient and deficient proteins.

The chief difficulties in conducting nutrition experiments with ground whole corn as the sole source of protein is due to its relatively low percentage of protein. When the other essential components of the diet, namely, an adequate inorganic salt mixture, butter fat, and lard, are added, the percentage of protein is too low to expect normal growth.

The only abundant protein concentrates of corn on the market are commercial gluten meal and gluten feed. The gluten meal is obtained as a by-product in the manufacture of starch, dextrin, and syrup from corn. Approximately 600,000 to 700,000 tons of gluten meal and gluten feed are produced annually. The corn is steeped in water containing sulfur dioxide in order to soften it and is then ground under water and the mixture is strained through sieves to remove hulls, tip caps, and the embryos. The water containing the suspended starch and protein is passed over long troughs in which most of the starch settles. The protein passing out of the trough is filter-pressed and dried at a low temperature. This dried mixture is known commercially as gluten meal and is used extensively for making mixed cattle feeds. Gluten feed is made in the same manner but contains the residue obtained by evaporation of the steep water.

Several attempts have previously been made to grow white rats on corn gluten, prepared in various ways, as the sole source of protein. Osborne and Mendel have described nutrition experiments with gluten meal (2, 4). This gluten meal contained about 45 per cent of proteins. When this was the sole source of protein in the diet the rats maintained weight but made very little growth during an experiment which was continued for more than a year. The authors state that with this gluten meal (2) "more than very slow growth is impossible." When a part of the gluten meal was replaced by casein or lactalbumin normal growth was obtained. The inorganic salts and water-soluble vitamine were furnished by protein-free milk. The authors conclude their paper with the following statement: "It is perhaps not too utopian to expect that the day may come when amino-acid concentrates may serve to render perfect the mixtures of proteins in a fodder like maize or its commercial by-products." In a later publication (11) the same authors state that they have never yet been able to feed

a sufficient quantity of the proteins of the maize concentrate to effect normal growth although there is evidence that all the essential amino-acids are present in some part of the seed. They also state:

"Our experiments indicate that it ought to be possible to make an animal grow on a diet in which the maize kernel is the sole source of protein, provided a preparation of total proteins could be obtained which would permit feeding them in sufficient quantity so that enough of those amino-acids which are present in certain of the proteins and not in others would be available to meet the minimum nutritive requirements of the organism."

McCollum, Simmonds, and Pitz (5) have also attempted to grow rats on a diet containing various mixtures of separate parts of the corn kernel but were not successful in obtaining normal growth.

Hogan has made extensive studies of the nutritive value of corn and its proteins. In one of his experiments (7) he fed a mixture of corn and commercial corn gluten meal as the sole source of protein in the diet and obtained surprisingly good growth. He states that corn gluten seemed more efficient than either egg white or dried blood as a supplement to corn but adds that it is possible that this result should be confirmed by a larger number of experiments before the fact can be considered established. In a later experiment (8) he attempted to grow rats on a corn protein concentrate which he prepared himself in order that the embryo, which is absent in commercial corn gluten meal, might be present, but the attempts were not successful and some of the rats died relatively soon. In these experiments the percentages of corn proteins in the diet were only 9 or 12 per cent so that no definite conclusions can be drawn as to the value of the protein concentrate.

The purpose of our experiments with commercial gluten meal was not only to determine its nutritive value but also to endeavor to find some cheap concentrate that could be used to supplement the proteins of the gluten since zein, which constitutes approximately one-half of these proteins, is deficient in lysine and tryptophane. As will be seen in the description of the experiments given below this could be accomplished by the addition of coconut press-cake to the gluten meal. Normal growth was also obtained when a mixture of ground whole corn and gluten meal furnished the sole source of proteins in the diet. This diet contained 21 per cent of corn proteins.

Experiments with Gluten Meal as the Sole Source of Protein.—A diet was prepared which contained 53 per cent of gluten meal. This is equivalent to 19.4 per cent of protein. To this were added

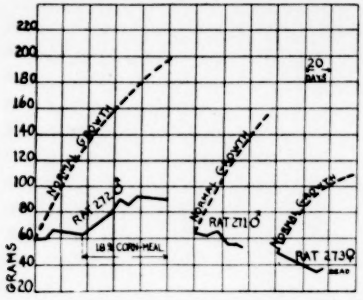

CHART 1. This chart shows the failure of rats to grow when gluten meal was the sole source of protein in the diet and no source of water-soluble vitamine was added. Rat 273 ♀ died in 36 days and the experiment was discontinued with Rat 271 ♂ at the end of that time, while 18 per cent of whole, ground, yellow corn replaced an equivalent amount of starch in the diet of Rat 272 ♂. This caused considerable growth for a short time; then growth ceased again. It is probable that Rat 272 ♂ was permanently injured by lack of water-soluble vitamine before the corn-meal was added. The composition of the diet before the adding of the ground corn was as follows:

	gm.	
Gluten meal	53	(Equivalent to 19.4 gm. of protein.)
Salt mixture*	4	
Starch	18	
Butter fat	18	
Lard	7	
	100	

* The composition of the salt mixture was as follows:

	gm.		gm.
$CaCO_3$	134.8	Citric Acid + H_2O	111.1
$MgCO_3$	24.2	Fe citrate + $1\frac{1}{2}$ H_2O	6.34
Na_2CO_3	34.2	KI	0.020
K_2CO_3	141.3	$MnSO_4$	0.079
H_3PO_4	103.2	NaF	0.248
HCl	53.4	$K_2Al_2(SO_4)_4$	0.0245
H_2SO_4	9.2		

Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 374.

a suitable salt mixture, starch, butter fat, and lard. Three rats were placed on this diet but none of them made any appreciable growth and one died at the end of 36 days. Rat 272 maintained its weight for 36 days and at the end of that time the 18 per cent of starch was replaced by whole, ground, yellow corn. Considerable growth took place but the rate was below normal and soon ceased.

There were two factors either of which might be responsible for failure of the rats to grow on the diet consisting of gluten meal as the sole source of protein. It was not known whether or not the gluten meal furnished proteins adequate for normal growth and if the necessary water-soluble vitamine was present. This accessory, which is necessary for growth, goes into the steep water. Since this was not incorporated in the gluten meal used in our experiments, it seemed probable that the failure to obtain growth was due to the lack of water-soluble vitamine. The curves obtained in these experiments are shown in Chart 1.

Experiments with Gluten Meal and Brewers' Yeast.—The diet used in these experiments contained 53 per cent of gluten meal and 3 per cent of dried brewers' yeast together with a salt mixture, starch, butter fat, and lard. Since brewers' yeast has been shown to contain an abundance of water-soluble vitamine (12) any failure to grow could not be attributed to the lack of this dietary essential. Two rats were placed on this diet and as is shown by the curves in Chart 2 the rate of growth was above normal which was surprising in view of the results obtained by other investigators. It is very evident that the yeast furnished an adequate supply of water-soluble vitamine. Since yeast also contains proteins adequate for normal growth (13), the success obtained in this experiment might be partly due to the proteins of the yeast although it seemed improbable that the small quantity of proteins contributed by 3 per cent of yeast could have much effect on the rate of growth.

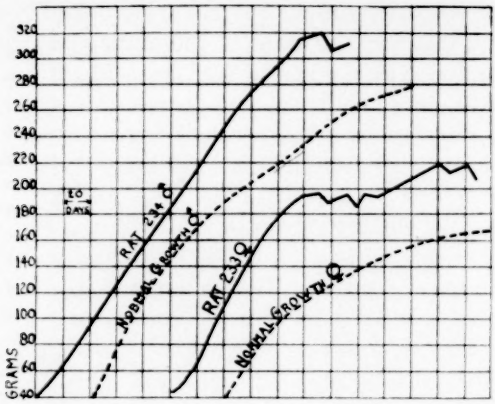

CHART 2. This shows excellent growth when gluten meal was the sole source of protein and the water-soluble vitamine was furnished by 3 per cent of dried brewers' yeast. With this exception the diet was similar to the one on which rats failed to grow (Chart 1). It is apparent that gluten meal contains a mixture of proteins adequate for normal growth. This contention is also borne out by the results shown by the curves in Chart 3 in which the water-soluble vitamine was furnished by 18 per cent of whole, yellow corn-meal.

The composition of the diet was as follows:

	gm.	
Gluten meal	53	(Equivalent to 19.4 gm. of protein.)
Salt mixture	4	
Starch	15	
Dried brewers' yeast	3	
Butter fat	18	
Lard	7	
	100	

Experiments with a Mixture of Gluten Meal and Corn-Meal as the Sole Source of Protein and Water-Soluble Vitamine.—This diet contained 53 per cent of gluten meal and 18 per cent of yellow corn-meal made from the whole corn. Two rats were placed on this diet and their growth was remarkably good, the rate of growth of the female being normal. These results confirmed those obtained by Hogan (7, 8) on a similar diet and show that corn proteins of sufficient concentration in the diet are adequate for normal growth. It is also evident that 18 per cent of corn-meal

furnishes an adequate supply of water-soluble vitamine. It is probable, however, that the vitamine content of this diet was very near the minimum quantity required. The curves obtained in these experiments are shown in Chart 3.

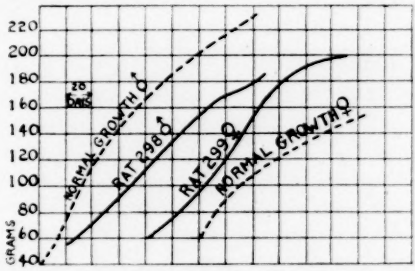

CHART 3.—The curves in this chart show that normal growth can be obtained from a diet in which a mixture of gluten meal and ground corn furnishes the sole source of protein and water-soluble vitamine. The gluten meal contributed 19.4 per cent of protein in the diet while the ground corn contributed only 1.5 per cent. The water-soluble vitamine contributed by the ground corn was probably near the minimum requirement of this food accessory. The composition of the diet was as follows:

	gm.	
Gluten meal	53	(Equivalent to 19.4 gm. of protein.)
Salt mixture	4	
Whole ground corn	18	(Equivalent to 1.5 gm. of protein.)
Butter fat	18	
Lard	7	
	100	

Experiments with Mixtures of Gluten Meal and Coconut Press-Cake.—Although normal growth can be obtained from a diet in which concentrated corn proteins furnish the sole source of proteins, such a diet is probably not economical because approximately one-half of these proteins are deficient in lysine and tryptophane. It therefore seemed desirable to find a cheap protein concentrate which when mixed with gluten meal would contribute sufficient quantities of lysine and tryptophane to constitute an efficient mixture of proteins. Our previous work on the nutritive value of the proteins of coconut press-cake (14) suggested that this substance might be used advantageously for this pur-

pose. A diet was, therefore, prepared in which 25 per cent of corn gluten and 25 per cent of coconut press-cake furnished the sole source of protein. This diet contained 14 per cent of protein (N × 6.25) of which approximately 9 per cent was furnished by corn gluten and 5 per cent by coconut press-cake. The rest of the diet consisted of a salt mixture, starch, butter fat, and lard.

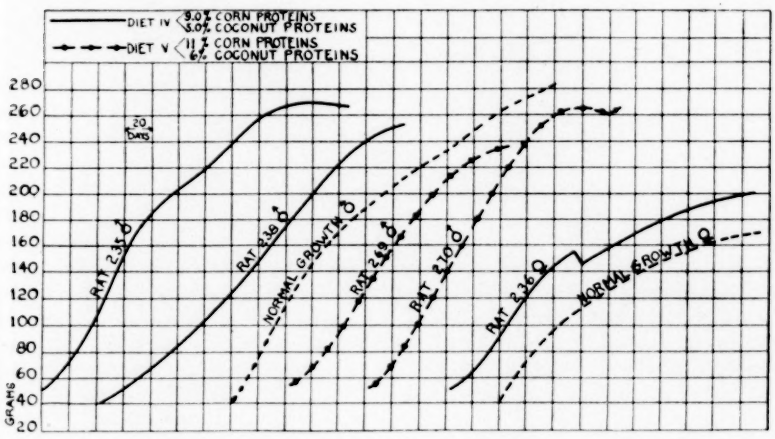

CHART 4. These curves show that excellent growth can be obtained when a mixture of gluten meal and coconut press-cake furnishes the sole source of protein and water-soluble vitamine in the diet. The high efficiency of this mixture is indicated by the results obtained on Diet IV which contained but 14 per cent of proteins of which only 5 per cent was furnished by the coconut press-cake. This indicates the value of coconut press-cake as a supplement to corn proteins. The compositions of Diets IV and V are as follows:

	Diet IV.	Diet V.
	gm.	*gm.*
Gluten meal	25	30
Coconut press-cake	25	31
Salt mixture	4	4
Starch	21	10
Butter fat	10	18
Lard	15	7
Total	100	100

The rats (Nos. 235, 238, and 236) fed on this diet grew normally, indicating an efficient mixture of proteins. It is also evident that 25 per cent of coconut press-cake furnished sufficient water-soluble vitamine for normal growth. A second diet containing 30 per cent of gluten meal and 31 per cent of coconut press-cake also gave excellent results. The growth obtained in these experiments is shown by the curves in Chart 4. Hence, it seems practical and economical to supplement gluten meal with coconut press-cake both for the purpose of supplementing the proteins of gluten meal and adding the water-soluble vitamine which gluten meal lacks.

SUMMARY.

1. Commercial corn gluten meal supplemented by dried brewers' yeast, whole, ground, yellow corn, or coconut press-cake furnishes the necessary protein for normal growth.

2. 18 per cent of whole, ground, yellow corn-meal furnishes sufficient water-soluble vitamine for normal growth.

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THE EQUILIBRIUM BETWEEN OXYGEN AND CARBONIC ACID IN BLOOD.

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I.

After many years of uncertainty, a very high degree of probability seems to have been established for the hypothesis that the absorption of oxygen by blood depends upon a chemical combination between oxygen and hemoglobin, $Hb + O_2 = HbO_2$, in which the molecular weight of hemoglobin has the lowest possible value, so that 1 atom of iron corresponds to 1 molecule of oxygen in the compound oxyhemoglobin.

This chemical equilibrium has been very thoroughly studied by Barcroft and his associates (1). As a result of their investigations it is known that pure solutions of hemoglobin combine with oxygen in such a manner that the requirements of the simple mass law equation

$$k = \frac{[Hb] \cdot [O_2]}{[HbO_2]} \qquad (1)$$

are fulfilled. But when electrolytes are also present in the solution this relation is modified. Under such circumstances Barcroft has found that an equation developed by Hill[1]

$$k = \frac{[Hb] \cdot [O_2]^n}{[HbO_2]} \qquad (2)$$

expresses the conditions. In this equation, however, the values of n and k vary with the nature and concentration of the electrolytes. For human blood the value of n is always approxi-

[1] Barcroft (1), p. 60.

mately 2.5, and the expression becomes

$$k = \frac{[\mathrm{Hb}] \cdot [\mathrm{O_2}]^{2.5}}{[\mathrm{HbO_2}]} \tag{3}$$

The theoretical significance of this expression, in spite of many cogent arguments, remains somewhat obscure. This question will be further considered below.

With constant temperature, the principal variation in the value of k in human blood depends upon variations of the concentration of free carbonic acid, a quantity which is determined

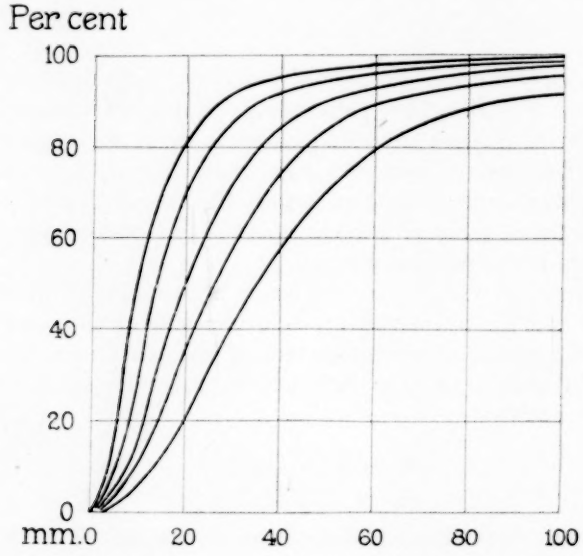


FIG. 1. Oxygen dissociation curves of Barcroft's blood, reading from left to right, exposed to 0, 3, 20, 40, and 90 mm. of CO_2. Ordinates are percentage saturation; abscissæ oxygen pressures in mm. of Hg.

by, and proportional to, the tension of carbon dioxide. This is the phenomenon discovered by Bohr, Hasselbalch, and Krogh (2). These conditions are illustrated by dissociation curves of Barcroft's blood[2] (Fig. 1).

For the curves of Fig. 1 the values of k (corresponding to $\frac{1}{K}$

[2] Barcroft (1), p. 65.

in Barcroft's notation) are as follows:

CO_2, *mm*	0	3	20	40	90
k	388	770	1,980	3,430	7,420

It may readily be seen, when the large variations in the values of k are noted, that we have no great assurance that the value of k for a single tension of carbon dioxide, *i.e.* for a single curve, is not affected with secondary variations, especially near the ends of the curves.

The simplicity of the mathematical expression also suggests that it should be possible to express the value of k in terms of $[CO_2]$, the tension of carbon dioxide, and thus to obtain the equation of the surface whose axes are percentage saturation with oxygen, oxygen pressure, and carbon dioxide pressure respectively. This expression turns out to be as follows:

$$\frac{[CO_2] + 7.7}{0.014} = \frac{[Hb] \cdot [O_2]^{2.5}}{[HbO_2]} \qquad (4)$$

For, putting $k = \frac{[CO_2] + 7.7}{0.014}$ we get the following values:

$[CO_2]$, *mm*	0	3	20	40	90
k	550	764	1,980	3,410	6,980

These values correspond to those of Barcroft within 1 per cent throughout the greater part of the range of carbon dioxide tension, and differ sensibly only in case carbonic acid is entirely absent, a peculiarity which, on theoretical grounds, is to be expected.

When Equation 4 is written

$$\frac{[CO_2] + 7.7}{0.014 \cdot [O_2]^{2.5}} = \frac{[Hb]}{[HbO_2]}$$

it is evident, from general thermodynamical principles, since increasing the concentration of carbon dioxide results in an increase of the concentration of oxygen, that increasing the tension of oxygen must likewise increase the tension of carbon dioxide. This conclusion, as far as I can see, could fail only in case there were present something resembling a ratchet mechanism, by which the carbon dioxide could act upon the oxygen without the possibility of a corresponding reaction. But such mechanisms are at present unknown in physicochemical systems.

However this may be, the investigation of Christiansen, Douglas, and Haldane (3) has proved that oxygen does influence the tension of carbon dioxide in blood in the manner indicated by the theory.

II.

In blood all the carbonic acid is present either as free acid ($CO_2 + H_2CO_3$) or as bicarbonate ($HCO_3 + BHCO_3$). This conclusion, originally reached from a study of the general acid-base equilibrium of the blood (4, 5), has been verified with great accuracy by the investigation of Hasselbalch (6). If we express the concentration of free carbonic acid by $[CO_2]$ and the concentration of total bicarbonate including both undissociated molecules and the bicarbonate ions by $[BHCO_3]$, we may write the expression for the equilibrium between these substances in the approximate form

$$k_{CO_2} = [H^+] \frac{[BHCO_3]}{[CO_2]} \qquad (5)$$

Here k_{CO_2} is a constant having for blood a value appreciably greater than that of the ionization constant of carbonic acid.

Equations 4 and 5 define the conditions for equilibrium between the hydrogen ion, carbonic acid, oxygen, hemoglobin, and bicarbonates in human blood. They are known to be true, at least with a fair approach to accuracy, on purely experimental grounds, independently of all theoretical considerations.

Several important conclusions follow directly from Equations 4 and 5. All of these, however, may be more clearly understood with the help of further theoretical considerations.

III.

The influence of carbonic acid upon the equilibrium between oxygen and hemoglobin must depend upon a chemical reaction in which both hemoglobin and carbonic acid are involved. Let us first consider the direct combination whereby hemoglobin bicarbonate is formed. For this purpose we may use Bohr's investigation (7) of the absorption of carbon dioxide by purified hemoglobin solutions.

From his numerous measurements Bohr drew the conclusion that the amount of carbonic acid united with hemoglobin varies with the tension of carbon dioxide so that, within those ranges of tension which exist in the blood, it amounts to about 0.5 cc. of CO_2 per gm. of hemoglobin. Bohr's experiments, however, were carried out in the absence of inorganic bicarbonates. Accordingly, the hydrogen ion concentration must have been very much higher than in blood. It follows that the amount of carbonic acid united to hemoglobin in blood must be at most very small, probably not more than 1 or 2 per cent by volume, and the variation of this quantity must be practically neglible.[3] This subject will be discussed below.

Moreover, as Bohr showed (8), the union of carbonic acid with hemoglobin is uninfluenced by the presence of oxygen, so that this process cannot be involved in the phenomenon with which we are now concerned. In fact, as Bohr pointed out (8), it must be the globin portion of the hemoglobin molecule which combines with carbonic acid.

Secondly, we may examine the problem of the distribution of a base between carbonic acid and hemoglobin functioning as an acid. This seems to be the only remaining possibility of explaining the interaction of oxygen and carbon dioxide. It follows from this consideration, as suggested by Hasselbalch and Lundsgaard (9), and by Christiansen, Douglas, and Haldane (3), and then more strongly asserted by Hasselbalch (6), and by Parsons (10), that oxyhemoglobin must be a stronger acid than reduced hemoglobin. Of course it also follows that the salts of hemoglobin must have a greater affinity for oxygen than has acid hemoglobin itself. Accordingly we seem to be concerned with the following different reactions:

$$\begin{aligned}
HHb &+ NaHCO_3 = NaHb + H_2CO_3 \\
HHb &+ O_2 = HHbO_2 \\
NaHb &+ O_2 = NaHbO_2 \\
HHbO_2 &+ NaHCO_3 = NaHbO_2 + H_2CO_3
\end{aligned}$$

Moreover, all the substances involved in these reactions, except oxygen, ionize in a greater or less degree. In order to study this

[3] I discussed this question with Professor Bohr at the Heidelberg Congress in 1907.

question we may turn to the data of Christiansen, Douglas, and Haldane (3), from which the first four columns of Table I are constructed by means of the curves on page 256 of their paper. Column 1 gives carbon dioxide pressures in mm.; Column 2 the corresponding concentrations of free carbonic acid in volumes per cent; Column 3 the total carbonic acid absorption of the blood when equilibrium is established in the presence of oxygen, so that practically all the hemoglobin is in the form of oxyhemoglobin; Column 4 the corresponding value when oxygen is absent, so that all the hemoglobin is in the reduced condition. From these values those of Column 5 = Column 3 − Column 2 and

TABLE I.

(1) CO_2	(2) $[CO_2]$	(3) Total CO_2. O.*	(4) R.*	(5) $[BHCO_3]$ O.	(6) R.	$\overset{+}{[H]} \times 10^9$N	
						(7) O.	(8) R.
mm.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*		
5	0.34	20.0	24.0	19.7	23.7	1.28	1.09
10	0.67	28.5	32.5	27.8	31.8	1.86	1.67
20	1.34	38.5	43.5	37.2	42.2	2.92	2.61
30	2.02	46.5	51.5	44.5	49.5	3.78	3.43
40	2.69	51.5	57.0	48.8	54.3	4.62	4.30
50	3.36	56.0	62.0	52.6	58.6	5.44	4.95
60	4.03	59.5	66.0	55.5	62.0	6.20	5.65
70	4.70	63.0	69.5	58.3	64.8	6.93	6.30
80	5.38	66.5	73.0	61.1	67.6	7.63	6.97

* O = oxidized blood; R = reduced blood.

Column 6 = Column 4 − Column 2 are calculated. These give the combined carbonic acid of the fully oxygenated and of the fully reduced blood. Columns 7 and 8 give the corresponding values of hydrogen ion concentration in accordance with Equation 5 and the research of Hasselbalch (6). Column 7 refers to the oxidized and Column 8 to the reduced blood.

In Fig. 2 the values of Columns 5 and 6 are plotted against those of Columns 7 and 8, thus illustrating the change in combined carbonic acid which accompanies change in hydrogen ion concentration in fully oxidized and fully reduced blood, respectively.

Points on these two curves having the same abscissa are isohydric points. Accordingly the difference of their ordinates represents the amount of base transferred from carbonic acid to hemoglobin when fully reduced blood is fully saturated with

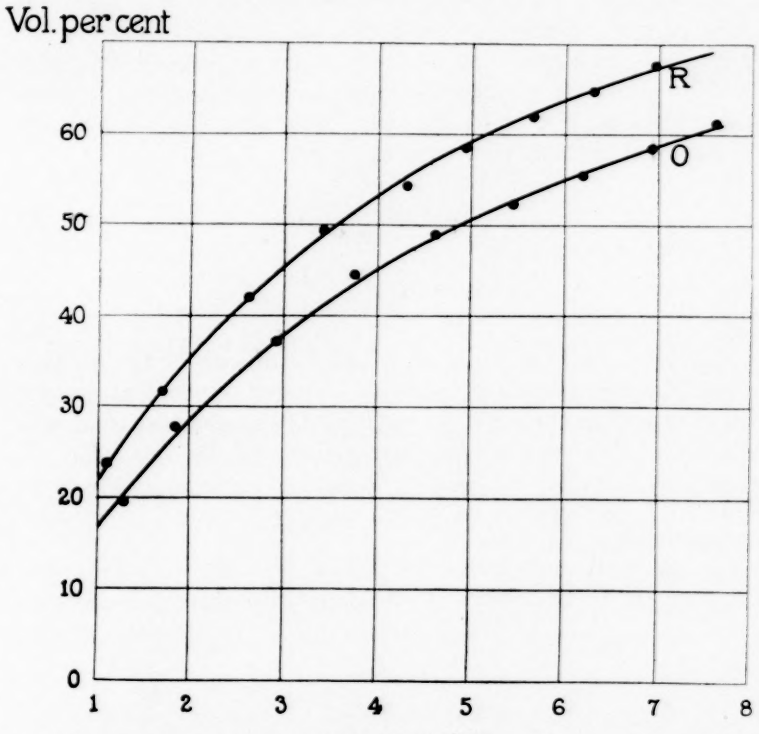


FIG. 2. Combined CO_2 as a function of $[H^+]$. Upper curve fully reduced blood; lower curve fully oxygenated blood of J. S. H. Ordinates are combined CO_2 in volumes per cent; abscissæ $[H^+] \times 10^8$N.

oxygen isohydrically; *i.e.*, in such a manner, by regulating the tension of carbon dioxide, as to keep the hydrogen ion concentration constant. The only acids that can be involved in this reaction are hemoglobin and carbonic acid, because while the hydrogen ion concentration remains constant other acid substances can neither combine with nor liberate base. But car-

bonic acid can do this if the tension of carbon dioxide is varied, and hemoglobin can do it if, by combining with oxygen, it becomes a stronger acid. Fig. 2 therefore affords a rigorous proof of the variation of the affinity of hemoglobin for base with variation in the amount of oxygen combined with hemoglobin. Thus the study of the isohydric properties of the system enables us to draw conclusions regarding the acid nature of hemoglobin. For the acid radical of hemoglobin which increases in strength when oxyhemoglobin is formed we may write mass law equations analogous to Equation 5 for carbonic acid.

$$k_R = [\overset{+}{\mathrm{H}}] \frac{[\mathrm{BHb}]}{[\mathrm{HHb}]} \tag{6}$$

$$k_O = [\overset{+}{\mathrm{H}}] \frac{[\mathrm{BHbO_2}]}{[\mathrm{HHbO_2}]} \tag{7}$$

It will be convenient to express concentrations of the various hemoglobin compounds in units of volumes per cent of oxygen and CO_2, noting that [BHb] and [$BHbO_2$] stand for the total concentrations, both ionized and non-ionized, of the two salts. If we assume that the total concentration of hemoglobin for the blood of J. S. H. corresponds to 18 per cent by volume of oxygen or carbon dioxide and write

$$[\mathrm{BHbO_2}] - [\mathrm{BHb}] = [\mathrm{BHCO_3}]_R - [\mathrm{BHCO_2}]_O = \Delta s$$
$$[\mathrm{BHb}] = s$$

we find

$$[\mathrm{HHb}] = 18 - s$$
$$[\mathrm{BHbO_2}] = s + \Delta s$$
$$[\mathrm{HHbO_2}] = 18 - s - \Delta s$$

values which hold for isohydric solutions both inclusively and exclusively.

The mass law equations then become

$$k_R = [\overset{+}{\mathrm{H}}] \frac{s}{18 - s} \tag{8}$$

$$k_O = [\overset{+}{\mathrm{H}}] \frac{s + \Delta s}{18 - s - \Delta s} \tag{9}$$

In order to solve these equations it is necessary to obtain two sets of values for $[\overset{+}{H}]$ and Δs. These may be obtained from Fig. 2, for example,

$$[\overset{+}{H}]_1 = 2 \times 10^{-8} \quad \Delta s_1 = 6.7$$
$$[\overset{+}{H}]_2 = 6 \times 10^{-8} \quad \Delta s_2 = 8.8$$

whence

$$s_1 = \frac{27\, s_2}{9 + s_2}$$

$$\frac{s_1 + 6.7}{11.3 - s_1} = \frac{3\, s_2 + 26.4}{9.2 - s_2}$$

and

$$s_1 = 9.64$$
$$s_2 = 5.00$$

Using these values we find for the two cases in question the values of Table II.

TABLE II.

$[\overset{+}{H}]_1 = 2 \times 10^{-8}$	$[BHb]_1 = 9.64$	$[BHbO_2]_1 = 16.34$	$k_R =$	2.3×10^{-8}
	$[HHb]_1 = 8.36$	$[HHbO_2]_1 = 1.66$	$k_O =$	19.7×10^{-8}
$[\overset{+}{H}]_2 = 6 \times 10^{-8}$	$[BHb]_2 = 5.00$	$[BHbO_2]_2 = 13.80$	$k_R =$	2.3×10^{-8}
	$[HHb]_2 = 13.00$	$[HHbO_2]_2 = 4.20$	$k_O =$	19.7×10^{-8}

From these values of k_R and k_O and Equations 8 and 9 it is possible to calculate all isohydric values of [BHb] and [$BHbO_2$], and therefore of Δs. Some of these are given in Table III.

TABLE III.

(1) $[\overset{+}{H}] \times 10^8 N$	(2) [BHb]	(3) [$BHbO_2$]	(4) Δs = (3) − (2)
	vol. per cent	*vol. per cent*	*vol. per cent*
1	12.5	17.1	4.6
2	9.6	16.3	6.7
3	7.8	15.6	7.8
4	6.6	15.0	8.4
5	5.6	14.4	8.8
6	5.0	13.8	8.8
7	4.5	13.3	8.8
8	4.0	12.8	8.8
9	3.7	12.4	8.7

It may readily be seen that these values of Δs approximately correspond with the differences in the ordinates of the two curves of Fig. 2. Only in the neighborhood of $[\overset{+}{H}] = 1 \times 10^{-8}$ is there a significant discrepancy. But since the shape of the curves of Christiansen, Douglas, and Haldane is uncertain in this range ($[CO_2] < 10$ mm.) it is impossible to attach any significance to this difference. On the other hand it must be carefully noted that the relative values of k_R and k_O depend upon differences between the several values of Δs obtained from the data of Christiansen, Douglas, and Haldane. These differences are highly uncertain. Accordingly it is hardly worth while to attempt accurately to determine the absolute magnitude of the constants. Nevertheless it is easy to show mathematically that the range of this variation consistent with the general character of the data of Christiansen, Douglas, and Haldane is very limited. Accordingly, we may say that the order of magnitude of these quantities is given by the equations

$$k_R = 2.3 \times 10^{-8} \tag{10}$$

$$k_O = 2.0 \times 10^{-7} \tag{11}$$

IV.

While the considerations above set forth afford a possible, and, as I believe, the real explanation of the influence of oxygen upon the absorption of carbon dioxide by blood, it is evident that the fluctuation in the amount of alkali combined with a single acid radical of hemoglobin is far from sufficient to account for the variation of $[BHCO_3]$ with varying $[\overset{+}{H}]$. The facts are illustrated in Table IV. Column 4 gives the quantities of base that can be accounted for, on the above considerations, in the blood at the hydrogen ion concentrations indicated in Column 1. It will be seen that for $[\overset{+}{H}] = 7 \times 10^{-8}$N basic radicals chemically equivalent to about 70 per cent by volume of carbon dioxide are in evidence, while at a hydrogen ion concentration one-fourth as great an amount equivalent to only 40 per cent by volume is accounted for. What has become of the remainder? In some measure basic radicals have become free, as in the case of a portion of those of globin which were directly united with carbonic acid.

This is, however, a negligibly small factor. The great majority of the missing basic radicals have combined with other weak acid radicals. Thus a large fraction of whatever phosphoric acid may be present has been converted from molecules of the type BH_2PO_4 to B_2HPO_4. But this again represents a small fraction of the total.[4] Probably a considerable number of other substances are more or less involved in this same manner. But in a considerable degree it must be the acid radicals of the proteins which are in question. Of these there is an indefinitely large supply. They are present in the protein portion of the hemoglobin molecule and in all the proteins of the plasma. In short we come back to the old reaction, so often discussed,

$$BHCO_3 + H\ Protein \rightleftarrows H_2CO_3 + B\ Protein$$

TABLE IV.

(1) $+$ $[H] \times 10^8 N$	(2) $[BHCO_3]_0$	(3) $[BHbO_2]$	(4) $B = (2) + (3)$
	vol. per cent	*vol. per cent*	*vol. per cent*
2	29.6	16.3	46
3	38.0	15.6	54
4	44.8	15.0	60
5	50.3	14.4	65
6	54.9	13.8	69
7	58.5	13.3	72

It is important to consider this reaction because there is danger of confounding the unique case of hemoglobin above discussed with the universal property of all proteins and so overlooking or misconceiving the latter.[5] Moreover the acid radical of hemoglobin which has been in question is not only unique in its properties, it is probably not even a protein radical at all, but a radical of the hematin part of the molecule.

[4] My original discussion of the theory of neutrality regulation (4, 5) has often been misunderstood at this point. I have not intended to imply that phosphates are important in the regulation of the reaction of the blood, unless in blood cells of species where much phosphoric acid is present, but rather in the body as a whole, *i.e.* in the tissues, and in the process of excretion.

[5] This appears to be the only source of difference between certain conclusions which Parsons has reached (10) and the corresponding conclusions of this paper.

Every protein molecule contains a considerable number of acid and basic radicals. The researches of Sörensen (12) show that these radicals obey the ordinary laws of chemical equilibrium and my own experience with gluten (13) indicates that they conform to the simple laws with considerable accuracy, even under the most varied colloidal conditions. But since the several acid radicals of a protein possess different ionization constants the behavior of a protein, as the hydrogen ion concentration varies, corresponds, not to that of the solution of a single weak acid, but to that of a mixture of a considerable number of weak acids. Thus, if we calculate an ionization constant in the usual manner, we find that this varies with the hydrogen ion concentration. Nevertheless the change in this apparent ionization constant for small changes of hydrogen ion concentration is small and it ought not to be difficult roughly to test the question whether the protein may probably be accounted the cause of a considerable part of the variation in $[BHCO_3] + [BHbO_2]$ with varying $[\overset{+}{H}]$.

From Table IV it may be seen that on the assumption that proteins alone are involved, the variation in the amount of base in union with protein in blood between $[\overset{+}{H}] = 1 \times 10^{-8}$N and $[\overset{+}{H}] = 1 \times 10^{-7}$N must be equivalent to about 50 per cent by volume of carbonic acid. Such a variation of the quantity of base would correspond, in round numbers, to 2 mols of base per mol of protein in the blood as a whole, assuming that the other proteins average the same molecular weight as hemoglobin. In other words, while passing from $[\overset{+}{H}] = 1 \times 10^{-8}$N to $[\overset{+}{H}] = 1 \times 10^{-7}$N, somewhat less than 10,000 gm. of the mixed protein of the whole blood must give up 1 mol of base, if the phenomenon is ascribed to the basic radicals of the proteins alone.

It is easy to see that if, on the average, every 500 gm. of blood protein contains 1 mol of acid radical of such a nature that in the range between $[\overset{+}{H}] = 1 \times 10^{-8}$N and $[\overset{+}{H}] = 1 \times 10^{-7}$N all these radicals together behaved approximately like so many acid radicals of the strength $k = 5 \times 10^{-10}$ the necessary condition would be fulfilled. This appears to be a possible assumption, although it is not impossible that the proteins are on the whole even weaker acids and that small quantities of other substances

such as weak organic acids or organic compounds of phosphoric acid may be involved in a small degree. For comparison it may be noted (13) that about 30,000 gm. of gluten are required to liberate 1 mol of base between $[\overset{+}{H}] = 1 \times 10^{-8}$N and $[\overset{+}{H}] = 1 \times 10^{-7}$N. In the case of the serum proteins (14, 15) the necessary amount of protein is only about 15,000 gm. It must be remembered, however, that globin differs from these.

Certain observations of Hasselbalch,[6] although inconclusive, suggest that the acid radicals of globin may be strong enough to account for nearly the whole change within the corpuscles. The isoelectric point of hemoglobin suggests the same conclusion.

In any case it is evident that the facts are consistent with the general theory of acid-base equilibrium and the well established properties of proteins. The newly discovered property of hemoglobin does not lead to new difficulties, but, on the contrary, removes at least one serious difficulty of long standing. It is to illustrate this fact that the present section has been written, and no claim is made whatever for the accuracy of the estimates which are involved.

V.

Equations 6 and 7 yield the equation

$$\frac{k_O}{k_R} = \frac{[BHbO_2] \cdot [HHb]}{[BHb] \cdot [HHbO_2]} \tag{12}$$

For infinite dilution the mass law equations for the reactions

$$BHbO_2 = BHb + O_2$$
$$HHbO_2 = HHb + O_2$$

have the simple form

$$k_S = [O_2] \frac{[BHb]}{[BHbO_2]} \tag{13}$$

$$k_A = [O_2] \frac{[HHb]}{[HHbO_2]} \tag{14}$$

whence

$$\frac{k_A}{k_S} = \frac{[BHbO_2] \cdot [HHb]}{[BHb] \cdot [HHbO_2]} \tag{15}$$

[6] Hasselbalch (6), p. 130.

and from Equations 12 and 15

$$\frac{k_A}{k_s} = \frac{k_O}{k_R} \tag{16}$$

Finally this expression, together with the approximations of Equations 10 and 11, gives

$$\frac{k_A}{k_s} = 9 \tag{17}$$

In spite of the fact that Equation 17 is a very rough approximation, it will be convenient to employ it as a means of estimating the probable effect that carbonic acid exerts upon the

TABLE V.

(1) $+$ $[H] \times 10^8 N$	(2) [BHb]	(3) [HHb]	(4) k_1 Calculated.	(5) K Barcroft.	(6) (5)/(4)
	vol. per cent	*vol. per cent*			
1	12.5	5.5	3.6	764	210
2	9.6	8.4	5.1	1,265	250
3	7.8	10.2	6.0	1,980	330
4	6.6	11.4	6.6	2,770	420
5	5.6	12.4	7.0	3,620	520
6	5.0	13.0	7.4	4,560	620
7	4.5	13.5	7.7	5,560	720
8	4.0	14.0	7.9	6,700	850
9	3.7	14.3	8.1	8,000	990

affinity of hemoglobin for oxygen. In Table V, Columns 1 and 2 of Table III are reproduced, Column 3 gives values of [HHb] = 18 − [BHb], Column 4 gives values of the apparent constant $k = [O_2]\frac{[Hb]}{[HbO_2]}$ which these imply, according to the relation[7]

$$k = k_s \frac{[BHb]}{18} + 9\, k_s \frac{[HHb]}{18}$$

These calculated values of k correspond to the requirements of the theory for fully reduced blood at infinite dilution. The differences in the values are maxima, for as the blood takes up

[7] Note that $9k_s = k_A$.

oxygen there is increasingly less and less difference between the amounts of base in union with hemoglobin at any two hydrogen ion concentrations. Column 5 contains values of k (equal to $\frac{1}{K}$ of Hill's formula) obtained from Barcroft's data. The scales of the two k's differ and it is therefore necessary to compare their ratios, which are given in Column 6.

Evidently there is no agreement between k as calculated and the K of Barcroft and Hill, save that they vary in the same sense. But this is inevitable, since the original assumptions, on which the value of the calculated k depends, rest upon the phenomenon which finds expression in Hill's formula in an increase of k, *i.e.*, a decrease of Hill's K, with increase of $[H^+]$; *i.e.*, with increase of $[CO_2]$.

This is the first real difficulty which we have encountered. The difficulty is real, because, first, the value of $\frac{k_A}{k_s}$, though uncertain, seems to be far too small to account for the variation in Barcroft's K, and secondly because it is evident that Barcroft's K should vary in accordance with the variation of k as theoretically calculated. This may readily be seen by writing equations according to Hill for the dissociation of acid oxyhemoglobin and its salt.

$$k_A = \frac{[HHb] \cdot [O_2]^{2.5}}{[HHbO_2]}$$

$$k_s = \frac{[BHb] \cdot [O_2]^{2.5}}{[BHbO_2]}$$

Then, as before,

$$\frac{k_A}{k_s} = \frac{[BHbO_2] \cdot [HHb]}{[BHb] \cdot [HHbO_2]}$$

Therefore, if Hill's expression not only permits the accurate calculation of points on the oxygen dissociation curve of blood, but also represents the real mechanism of the reaction, it appears to follow that

$$\frac{k_A}{k_s} > 100$$

Indeed it would seem probable that this is far too low an estimate, because, throughout the greater part of the absorption curves, when oxygen has already caused a further union between hemoglobin and base the values of k may be expected to vary less and less between the curves of different tensions of carbon dioxide.

This consideration, moreover, casts a doubt upon the validity of Hill's curves as an expression of the real mechanism of the reaction. For, since the union of oxygen with hemoglobin must increase the union of hemoglobin with base and since, on general thermodynamical principles, this must in turn increase the affinity of hemoglobin for oxygen, it follows that, unless there is some compensatory process of an unknown nature, the value of k for a given tension of carbon dioxide must be itself a function of $[O_2]$ and therefore a variable. Thus it may be seen that the problem is somewhat more complex than is implied even by the searching analysis of Barcroft and Hill.

Returning to the discrepancy between the calculated values of k and those given by Barcroft and Hill, it may be said, on chemical grounds, that an increase in the affinity of the acid radical in question greater than that obtained by calculation, for example,

$$4 < \frac{k_A}{k_s} < 20$$

is hard to accept.

It seems necessary to suppose that this acid radical is located in the hematin portion of the molecule, and that its position is very close to that taken by oxygen when it combines with hemoglobin. If the position were α or β to that of an atom with which oxygen is united, a sensible effect upon the two affinities would correspond with general chemical experience. This is also the reason for assuming that a single acid radical is in question. It seems certain that this number must be very small. At a much greater distance analogy suggests that this effect is out of the question. Perhaps the difference of the ionization constants of propionic acid, $k = 0.000014$, and lactic acid, $k = 0.00014$, is worth consideration.[8] It must not be forgotten, however, that the heat of formation of lactic acid from propionic acid is much greater than the heat of formation of oxyhemoglobin from hemoglobin.

[8] This same comparison has been made by Parsons (10).

On the other hand it is to be considered that in the latter case 2 atoms of oxygen are involved. And of course it is not inconceivable, although probably it does not agree with conceptions now held, that the addition of oxygen takes place upon the very atom which binds the alkali also.

The data of Christiansen, Douglas, and Haldane seem to be inconsistent with the conceivable hypothesis that the dissociable oxygen is an essential part of this acid radical, for then the values of Δs would be much higher in the more alkaline than in the more acid range.

Finally, however, it seems almost certain that, if the difference between k_1 and k_2 were of the order of magnitude indicated by Hill's theory, every preparation of blood in which there is a considerable amount of oxygen in combination would have nearly the same affinity for base, and therefore for oxygen, as every other, regardless of the tension of carbon dioxide. If this is true, Hill's equation cannot be regarded as a valid expression of the real mechanism of the reaction, though I have no doubt of its validity as a means of calculating points on the absorption curves.

Stated in the most general terms, the work of Barcroft conclusively proves that, in the presence of electrolytes and therefore in blood, the active mass of hemoglobin is different from that value which can be calculated from the absorption of oxygen. If we take this conclusion for granted and abandon the attempt theoretically to account for it, just because we are thereby freed from a theoretical interpretation, we are able tentatively to take a further step. For it seems to be probable, and I think the constancy of Hill's n for all values of $[CO_2]$ strongly supports the view, that with a given percentage saturation of hemoglobin, regardless of the tension of carbon dioxide, the active masses of oxyhemoglobin and of reduced hemoglobin are more or less roughly constant. For example, it seems justifiable to assume that for all samples of blood which are just half saturated with oxygen approximately

$$\frac{[Hb]}{[HbO_2]} = \frac{[HHb] + [NaHb]}{[HHbO_2] + [NaHbO_2]} = \text{Constant}$$

though of course it is by no means equally possible to assume that

$$\frac{[Hb]}{[HbO_2]} = 1$$

If this is true the expression

$$k = [O_2] \frac{[Hb]}{[HbO_2]}$$

may be assumed to hold, with the qualification that we have at present no means of estimating the values of [Hb] or $[HbO_2]$, which now stand for the true active masses, or even of $\frac{[Hb]}{[HbO_2]}$. Nevertheless there is reason to suppose that the values of this ratio are constant for all cases where the saturation of the blood with oxygen has the same value, regardless of the tension of carbon dioxide.

This enables us to use Barcroft's curves for the calculation of values of k which, for any given degree of saturation of the blood

TABLE VI.

$\frac{[Hb]}{[HbO_2]}$	9.00	2.33	1.00	0.43	0.11
$[CO_2]$	Oxygen tensions.				
mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
0	4	7	10	15	26
3	6	10	14	20	35
20	8	14	20	29	49
40	10	18	26	37	61
90	14	25	35	50	85

with oxygen, *i.e.* for any given ordinate, are probably comparable, though for different ordinates they are not comparable.

Table VI gives data taken from Fig. 1 of the values of $[O_2]$ in mm.

These data of Table VI yield the relative values of k given in Table VII. The calculation consists in dividing the oxygen tension for any particular degree of saturation and carbon dioxide tension by that for the same percentage saturation and 0 mm. of carbon dioxide tension.

It must be understood that the values of k given by Table VII are relative, and comparable only within each column of the table taken by itself. It is evident, however, that aside from errors in reading Barcroft's curves all the columns are identical, so that Table VII reduces to the very simple form of Table VIII.

Of course the identity of the columns of Table VII depends upon a mathematical property of Hill's equation, and the exact agreement cannot therefore be significant for the present discussion. But since Barcroft's curves agree, within the limits of experimental accuracy, with the observations, we are nevertheless justified in using Table VIII, which has just as much validity as the assumption that for any one percentage saturation the active masses of hemoglobin and of oxyhemoglobin are constant with varying tensions of carbon dioxide. And of course it has no more validity than this.

TABLE VII.

Values of k.

1.00	1.00	1.00	1.00	1.00
1.50	1.43	1.40	1.33	1.35
2.00	2.00	2.00	1.93	1.89
2.50	2.55	2.60	2.47	2.35
3.50	3.55	3.50	3.33	3.27

TABLE VIII.

$[CO_2]$	k
mm.	
0	1.0
3	1.4
20	2.0
40	2.5
90	3.4

We are now in a position to compare values of k obtained from Barcroft's data by an assumption which, I think, is certainly not less reasonable than that involved in Hill's equation, with values of k calculated from the values of k_A and k_s. For this purpose we may take the case of fully reduced blood as represented by Column 4 of Table V. Table IX gives the necessary values.

Column 4 of Table IX, giving the ratios of the two values of k, unlike the similar column of Table V, contains values which are roughly constant. I think we are therefore justified in saying that, after all, the influence of carbonic acid upon the affinity of blood for oxygen does probably correspond with the requirements

of the theory of acid-base equilibrium. At any rate there is certainly no decisive evidence of inconsistency.

Since Barcroft has proved the active masses of hemoglobin and oxyhemoglobin to be different from the apparent values, it is evident that there may be a discrepancy between the combination of hemoglobin with base as indicated by theory, and the actual facts. This consideration raises a doubt regarding the validity of the present quantitative conclusions in general, which the above calculation cannot wholly remove. It must be remembered, however, that there is much evidence to prove that under all sorts of colloidal conditions the union of proteins with bases and acids corresponds closely with the requirements of the mass law. We are therefore not only permitted but obliged to go as far with this theory as we can.

TABLE IX.

(1) $[CO_2]$	(2) k Table VIII.	(3) k Calculated.	(4) $\frac{(3)}{(2)}$
mm.			
3	1.4	3.8	2.7
20	2.0	5.7	2.9
40	2.5	6.7	2.7
90	3.4	7.8	2.3

Moreover, if we write equations as follows:

$$k_R = \overset{+}{[H]}^{2.5} \frac{[BHHb]}{[HHb]}$$

and

$$k_O = \overset{+}{[H]}^{2.5} \frac{[BHHbO_2]}{[HHbO_2]}$$

which correspond to Hill's assumption, it appears that the values

$$k_R = 7.8 \times 10^{-20}$$

$$k_O = 110 \times 10^{-20}$$

yield results which agree roughly with the facts and permit the same conclusions that have been deduced from the assumptions made in this paper. It will be seen that even here the value of

the ratio $\frac{k_O}{k_R}$ corresponds with what is to be expected on chemical grounds.

It remains true, however, that we have no satisfactory means of accounting for the fact that according to Hill's formula, at least in its original implication, the difference in affinity for oxygen at any two tensions of carbon dioxide is independent of the amount of combined oxygen. The doubts expressed by Barcroft,[9] concerning the validity of the theoretical curves in their upper ranges, nevertheless suggest a possible escape from this final complication.

VI.

Bohr's data (11) of the absorption of carbon dioxide by solutions of hemoglobin make possible a rough estimate of the extent to which hemoglobin combines with carbonic acid in blood. For example, we may make use of his results for a solution containing 3.8 per cent hemoglobin.

In Table X, Column 1 gives the tension of carbonic acid, Column 2 the dissolved free carbonic acid, Column 3 total dissolved carbonic acid, and Column 4 (Column 3 – Column 2) combined carbonic acid in cc. of gas for 37.8 cc. of solution. From these data Column 5 is calculated according to the expression

$$[\overset{+}{\mathrm{H}}] = 6 \times 10^{-7} \frac{[CO_2]}{[BHCO_3]}$$

The values of Column 5 represent minimum values for the hydrogen ion concentration, which are probably sensibly too low and which must therefore lead to an overestimate rather than to an underestimate of the combination of hemoglobin with carbonic acid in blood.

Extrapolation indicates that for $[\overset{+}{\mathrm{H}}] = 0.35 \times 10^{-7}\mathrm{N}$ the value of $[BHCO_3]$ should be about 0.75 cc., which agrees with the approximation that for low values of the hydrogen ion concentration, the value of $[BHCO_3]$ should be directly proportional to $[\overset{+}{\mathrm{H}}]$. This quantity is an estimate of the total quantity of acid which may be regarded as combined with hemoglobin functioning as a base at $[\overset{+}{\mathrm{H}}] = 0.35 \times 10^{-7}\mathrm{N}$ in 37.8 cc. of solution containing

[9] Barcroft (1), p. 59.

3.8 per cent hemoglobin. From this the total salt formation s of hemoglobin functioning as a base, expressed in units of per cent by volume, for blood containing 14 per cent hemoglobin may be obtained as follows:

$$s = \frac{0.75}{37.8} \times \frac{14}{3.8} \times 100$$

whence

$$s = 7.3 \text{ per cent by volume}$$

This estimate represents the sum of the concentrations of all undissociated salt molecules formed of hemoglobin with all the

TABLE X.

(1) CO_2	(2) $[CO_2]$	(3) Absorbed CO_2.	(4) $[BHCO_3]$	(5) $\overset{+}{[H]} \times 10^7 N$
mm.	*cc.*	*cc.*	*cc.*	
6.04	0.275	2.098	1.823	0.90
11.57	0.527	2.885	2.358	1.35
14.62	0.666	3.230	2.564	1.55
18.54	0.844	3.666	2.822	1.80
24.07	1.095	4.197	3.102	2.10
31.98	1.455	4.855	3.400	2.55
43.14	1.963	5.715	3.752	3.15
60.03	2.731	6.815	4.084	4.05
85.40	3.886	8.335	4.449	5.25
124.96	5.684	10.507	4.823	7.10
188.62	8.583	13.828	5.245	9.85

acids of the corpuscles, plus the concentration of hemoglobin cations. Since these salts include those of all acids, it seems safe to say that hemoglobin bicarbonate cannot amount to even one-third of the total. Roughly about 2 per cent by volume seems therefore to be the highest possible estimate. It is also evident that for changes of hydrogen ion concentration, which are physiologically significant, the change in concentration of hemoglobin bicarbonate is negligible.

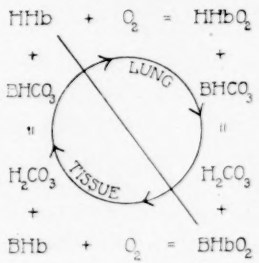
Experiments with blood involving wider changes of hydrogen ion concentration may, however, lead to sensible variations in the concentration of hemoglobin bicarbonate. Thus, for the range $\overset{+}{[H]} = 1 \times 10^{-8} N$ to $\overset{+}{[H]} = 1 \times 10^{-7} N$ the variation of hemo-

globin bicarbonate concentration is perhaps nearly 5 per cent by volume, and, as Table X shows, for higher values of $[H^+]$ this variation may be important. It must be noted, however, that such ranges of reaction cannot be reached except in the absence of the normal bicarbonates of the blood and in the presence of carbon dioxide at high tensions.

VII.

The changes which actually take place in the blood as a result of absorption of oxygen and escape of carbonic acid in the lung, followed by escape of oxygen and absorption of carbonic acid in the tissue, may now be considered. It is true that for this purpose we do not yet possess all the desirable experimental information, and it is evident that quantitatively the considerations developed in the preceding sections leave much to be desired. Nevertheless there seems to be little doubt about the general characteristics of the physiological process.

In the lung the escape of carbonic acid causes a transfer of base from this acid to the proteins, the phosphates, etc., and to the acid radical peculiar to hemoglobin. Meanwhile the absorption of oxygen leads to an increase in the concentration of oxyhemoglobin. Each of these processes facilitates the other, for while union of base with hemoglobin increases the affinity for oxygen the union of oxygen with hemoglobin increases the affinity for base. In the tissues these processes, and with them the affinity changes, are reversed. Such is the nature of the primary phenomenon, in accordance with the theoretical considerations of Sections III and V of this paper. An incomplete representation of the process is given by the accompanying diagram:



Certain cases of this cycle, which are indeed beyond the range actually occurring in the body, but which illustrate the principles involved, are defined by the data and the deductions above set forth.

Let us consider, for example, the case of blood in equilibrium, at a temperature of 38°, with an atmosphere consisting of ordinary air to which enough carbon dioxide has been added to make its tension 20 mm. This blood, as we have seen, will have approximately the following composition:

CO_2	Oxygen absorption.	$[BHbO_2]$	$[HHbO_2]$	$[BHCO_3]$	$[\overset{+}{H}]$
mm.	*per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
20	100	15.7	2.3	37.2	2.92×10^{-8}N

When this preparation is brought into equilibrium with an atmosphere free from oxygen in which the tension of carbon dioxide is 60 mm. the following conditions will be established:

CO_2	Oxygen absorption.	[BHb]	[HHb]	$[BHCO_3]$	$[\overset{+}{H}]$
mm.	*per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
60	0	5.3	12.8	62.0	5.65×10^{8}N

In such a case the effect of carbon dioxide upon the oxygen absorption is of course obscured. But, in default of other data, it is necessary to take instances where the oxygen absorption is either 0 or 100 per cent. Even here the *process* has, of course, been modified by the changing affinity of hemoglobin for oxygen. The defect is, however, not a serious one, since Barcroft's data (1), and those of Bohr, Hasselbalch, and Krogh (2) before him, sufficiently illustrate this point.

The increase in combined carbonic acid in the above imaginary experiment is evidently $62.0 - 37.2 = 24.8$ per cent by volume. This has been made possible by the liberation of an amount of base equivalent to $15.7 - 5.2 = 10.5$ per cent by volume from the acid radical peculiar to hemoglobin. The remainder of the base, equivalent to $24.8 - 10.5 = 14.3$ per cent by volume, has come from the proteins, including globin, and other buffer substances. This estimate is subject to a slight, but practically negligible, correction owing to the variation in the amount of hemoglobin bicarbonate.

If the hemoglobin did not vary in affinity for base, the increase in the amount of absorbed carbonic acid accompanying a change of hydrogen ion concentration from 2.92×10^{-8}N to 5.65×10^{-8}N would accordingly be only $\frac{14.3}{24.8} = 58$ per cent or roughly a little more than half the real change.

Again, if the hemoglobin did not vary in affinity for base the increase of the hydrogen ion concentration accompanying an increase of 24.8 per cent by volume in the combined carbonic acid would be more than 170 per cent of the real change. In other words the hydrogen ion concentration would have risen to more than 7.5×10^{-8}N.

Let us take two more conditions of equilibrium:

O_2	CO_2	Oxygen absorption.	[BHb]	[HHb]	[$BHbO_2$]	[$HHbO_2$]	[$BHCO_3$]	$[\overset{+}{H}]$
mm.	*mm.*	*per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
0	40	0	6.3	11.7			54.3	4.30×10^{-8}N
140	40	100			14.6	3.4	48.8	4.62×10^{-8}N

From these data we find that when oxygenated blood with the tension of carbon dioxide of 20 mm. is converted into reduced blood with a carbon dioxide tension of 40 mm. the hydrogen ion concentration changes from 2.92×10^{-8}N to 4.30×10^{-8}N. At the same time the carbonic acid increases by an amount equal to $54.3 - 37.2 = 17.1$ per cent by volume. The base yielded by the hemoglobin radical is $15.7 - 6.3 = 9.4$, and that yielded by ordinary buffer action, accordingly, $17.1 - 9.4 = 7.7$ per cent by volume.

Again, passing from oxygenated blood with a tension of carbon dioxide of 40 mm. to reduced blood with a tension of carbon dioxide of 60 mm. involves a change of hydrogen ion concentration from 4.62×10^{-8}N to 5.65×10^{-8}N; the increase of combined carbonic acid is $62.0 - 48.8 = 13.2$ per cent by volume; the base yielded by the hemoglobin radical is $14.6 - 5.2 = 9.4$ per cent by volume, and that yielded by ordinary buffer action only $13.2 - 9.4 = 3.8$ per cent by volume. In this last case, if the peculiar function of the hemoglobin were eliminated, the change in absorption of carbonic acid for the given change of hydrogen ion concentration would be only $\frac{3.8}{13.2} = 29$ per cent of its real value.

The change in the hydrogen ion concentration for the given change in combined carbonic acid, however, would be at least 350 per cent of the real value and the concentration of the hydrogen ion would rise to more than 8×10^{-8}N.

Since data defining all the conditions for tensions of oxygen corresponding to the physiological range are not at hand, this question can hardly be further analyzed.

From the above data, however, it is evident that the type of change which involves a very great absorption of carbon dioxide for a very small change in concentration of the hydrogen ion and, of course, a very small change in hydrogen ion concentration for a very large change in carbonic acid absorption is particularly marked for tensions of carbonic acid which are physiologically important. Moreover the shape of Barcroft's curves affords at least equally good evidence that it is also predominant within ranges of oxygen tension corresponding to those which exist in arterial and venous blood.

If this phenomenon is of the first importance as a means of increasing the efficiency with which oxygen and carbonic acid are carried to and from the tissues, it appears to be no less important as a factor in regulating the hydrogen ion concentration of the blood. This has been pointed out by Christiansen, Douglas, and Haldane (3), by Hasselbalch (6), and others.

The possibility of such a process as this, depending upon a cyclic variation in the ionization constant of an acid was completely, though I hope pardonably, overlooked in my original description of the acid-base equilibrium of the organism (4). And a claim that all the different types of physicochemical phenomena which are involved in the process had been at least considered (5) must accordingly now be withdrawn. I think there can be little doubt, however, that this is the only example of such a chemical mechanism which has ever come to light.

This leads to a further physiological consideration. It has long been an open secret that it was impossible theoretically to account for the quantity of carbonic acid which escapes from the blood in the lung. The paper of Christiansen, Douglas, and Haldane went far to overcome the difficulty, but the fact of the very slight variation of hydrogen ion concentration, which was the kernel of the whole question, remained or at least was insufficiently ex-

plained. In the light of the present considerations I believe that the difficulty probably disappears.

One of the most puzzling phenomena attending changes of the oxygen and carbon dioxide content of the blood is the increase in the volume of the red cells, accompanied by a passage of acid radicals from the plasma into the corpuscles, when carbonic acid tension increases. The process is of course reversed by a decrease of carbon dioxide tension. Some years ago Spiro and I succeeded in producing somewhat similar phenomena in much simpler systems (16). As a result of these experiments we reached the conclusion that the process depends, in the main, upon the fact that as carbonic acid concentration increases there is a shift of base from proteins to carbonic acid. If this explanation is true, it is natural to suppose that more base will be given up within the corpuscles than in the plasma, since the concentration of proteins in the corpuscles is much greater. As a result the osmotic pressure will increase more, and the hydrogen ion concentration less, in the corpuscles than in the plasma; water will pass into the corpuscles to reestablish the osmotic equilibrium, and acid to reestablish the acid-base equilibrium between the cells and the solution in which they are suspended.

Evidently this process helps to explain the escape of carbonic acid from the plasma without a change of the hydrogen ion concentration of the magnitude that would otherwise be necessary. In this way it was possible to show that the plasma proteins have no great share in controlling the reaction of the blood (17). But this consideration, while valid, obviates one difficulty only to raise another. This new difficulty, however, concerns the affinity of hemoglobin for base. But, in view of the basic character of the protein globin, I was at that time disposed to think that it was hard to account for so great a fluctuation in the amount of base combined with hemoglobin as was clearly indicated by even the roughest estimates of the magnitude of the process. Variation in the affinity of hemoglobin for base puts the whole question in a different light, and Hasselbalch and Warburg, who hold the same view of the general nature of the process, have pointed out that this goes far to explain the phenomenon (18). The theory developed in the present paper permits an estimate of the quantitative variations involved.

Let us turn once more to the change from blood saturated with air containing carbon dioxide at a tension of 40 mm. to the system in equilibrium with an atmosphere free from oxygen, in which the tension of carbon dioxide is 60 mm. There is involved an increase in combined carbonic acid amounting to 13.2 per cent by volume. Of this nearly three-quarters will come from the hypothetical hematin radical of variable affinity and, of the remaining one-fourth, the buffer action of globin and other substances within the corpuscles must account for much the greater share, leaving probably not more than 1 per cent by volume as the share of the buffer substances of the plasma. But, since the volume of the corpuscles is less than that of the plasma, the actual changes in concentration will be even more disparate.

For every molecule of bicarbonate thus added there must be a net increase of at least 0.5 molecule or ion in solution. Accordingly there must be a large increase in the osmotic pressure of the corpuscles and a very small increase in the osmotic pressure of the plasma. Similarly, there must be a relatively large increase in the hydrogen ion concentration of the plasma, or at least a tendency in this direction, and a very small increase in the hydrogen ion concentration of the corpuscles, because the increase in the hydrogen ion concentration is checked by the increase of bicarbonate according to the implications of the familiar equation

$$[\overset{+}{\mathrm{H}}] = k \frac{[\mathrm{CO_2}]}{[\mathrm{BHCO_3}]}$$

Taking these arguments into account it seems safe to say that the main features of the phenomenon are probably known.

In conclusion it needs to be said that the validity of the somewhat difficult, and no doubt quantitatively provisional, theoretical discussions of the present paper is not necessary for the validity of the conclusions. The influence of carbon dioxide on oxygen tension is a well established fact (1, 2). So is the influence of oxygen on carbon dioxide tension (3), and from these facts the influence upon the hydrogen ion concentration and the acid-base equilibrium follow necessarily (4, 5). Given these three postulates the other phenomena may be deduced. What is uncertain is the exact magnitude of these; their existence is, I believe, no longer open to question. It is important not to attach too much signif-

icance to estimates, necessarily provisional, of these magnitudes and I hope before long to be able to report results of a research, recently resumed after interruption by the war, which will permit a revision of the estimates of this paper.

SUMMARY.

This paper seeks to explain the interaction between oxygen and carbonic acid in blood by means of the theory of acid-base equilibrium.

The isohydric change from fully reduced to fully oxygenated blood; the transfer of base from carbonic acid to hemoglobin, which is the main feature of this process; and the change of strength as acid of a portion of the hemoglobin molecule, which is its cause, are discussed. It is shown that all these phenomena can be explained by the assumption that a certain acid radical of the hemoglobin molecule has, for reduced hemoglobin and oxyhemoglobin respectively, the following values:

$$k_R = 2.3 \times 10^{-8}$$

$$k_O = 2.0 \times 10^{-7}$$

From this consideration it follows that the salt of the acid radical in question must have a greater affinity for oxygen than the free acid. If the mass law constants of the reaction of salt and acid with oxygen were k_s and k_A respectively, it should be approximately true that

$$\frac{k_A}{k_s} = \frac{k_O}{k_R} = 9$$

In the light of these considerations the equilibrium between protein acid radicals and base in blood is examined.

The bearing of these considerations upon Hill's equation for the equilibrium between hemoglobin and oxygen is considered, but at this point the difficulties are only partially overcome.

The union of hemoglobin as a base with acids, and especially with carbonic acid in blood, is discussed.

Finally, the bearing of these considerations upon the physiological processes, both the homogenous reactions within the corpuscles and the plasma, and also the heterogenous exchanges between corpuscles and plasma, is investigated.

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FERMENTATION OF FRUCTOSE BY LACTOBACILLUS PENTOACETICUS, N. SP.*

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The fermentation of fructose by the pentose fermenters which have been called *Lactobacillus pentoaceticus, n. sp.*, presents some very interesting and unique aspects; *viz.*, the production of mannitol and the subsequent fermentation of this mannitol into acetic and lactic acids.

The production of mannitol by the fermentation of fructose has been observed by several investigators and has led these investigators to designate the fermenting organisms as "mannite bacteria;" *i.e.*, mannitol-forming bacteria.

Strecker was perhaps the first to obtain mannitol by a fermentation process. He allowed a mixture of hydrolyzed cane sugar, cheese, sour milk, and calcium carbonate to undergo spontaneous fermentation for 2 or 3 months. From the fermented mass he isolated mannitol in considerable quantities; *e.g.*, 1 pound of pure mannitol from the fermentation of 10 pounds of sugar. Dragendorff repeated the earlier work of Strecker and obtained a good yield of mannitol in his first fermentation, but a second experiment failed to show a trace of mannitol.

In the course of a discussion on the causes and conditions of lactic acid production Pasteur pointed out that besides lactic acid, other products, such as alcohol, butyric acid, and mannitol, were formed. He noted that great variations occurred in the amount of mannitol produced.

Marcano in the making of rum from the sugar-cane of the West Indies recognized mannitol as a product of the fermentation.

In all this early work no attempt was made to use pure cultures of bacteria or pure chemical substances.

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Gayon and Dubourg made a very careful study of the formation of mannitol in fermenting solutions. From a white wine they isolated an organism whose power of fermentation they tested on various sugars and related compounds. From fructose, mannitol was obtained in considerable quantity. Sucrose was readily fermented but gave no mannitol. On the other hand, when sucrose was first hydrolyzed into its two components glucose and fructose and then fermented, mannitol was formed. From this evidence Gayon and Dubourg concluded that sucrose was fermented directly by the bacteria and not inverted before fermentation, as is usually considered to be the case.

In an extensive study of the mannitic fermentation of wine Müller-Thurgau and Osterwalder isolated a number of organisms that produced mannitol from fructose. One of these, *Bacterium mannitopœum*, they report was able to ferment sucrose with the formation of mannitol. In two experiments crystals of mannitol were observed, but the amount formed was apparently too small to determine quantitatively as no separation of the crystals was made. Aside from these two experiments with *Bacterium mannitopœum*, no report has been found in the literature of mannitol formation directly from sucrose by means of a pure culture of bacteria.

Mannitol is frequently used as a source of carbon in the cultivation of bacteria. The ability of numerous organisms to break down mannitol is thus well established. The mannitol-forming bacteria as a group do not seem to possess this power. The organisms isolated and studied by Gayon and Dubourg, and by Müller-Thurgau and Osterwalder failed to grow on a medium in which fermentable sugars were replaced by mannitol.

Laborde isolated three organisms from wine which seemed to have the power of forming mannitol from fructose and later destroying the mannitol. He analyzed the cultures at two successive times in the course of the fermentation and obtained less mannitol in the second analysis than he had found 2 months earlier. His paper gives no data concerning the products formed from the mannitol.

All the mannitol-forming bacteria studied by the above investigators have been isolated from various kinds of wine. The organisms which are described in this and in our preceding papers (1919, 1920) have been isolated from different sources, such as manure, silage, and soil, and have a more general distribution in nature than the so called "mannite bacteria." *They are related to these bacteria by their ability to form mannitol from fructose; but a more conspicuous and characteristic property is their power to bring about a very rapid and complete fermentation of the pentose sugars xylose and arabinose.* It is probable that the general occurrence of the pentose fermenters is closely correlated with the

equally wide distribution of pentose-yielding compounds and that these bacteria play an important part in the decomposition of these plant compounds.

As will be shown later in this paper, the pentose fermenters produce mannitol in such quantities that it can be easily isolated, identified, and subsequently fermented by the organism that produced it.

The products formed by fermenting the mannitol have also been determined. The origin of this mannitol must be due to a reduction of fructose as a result of conditions incident to the fermentation process. On the basis of the experimental facts developed in this paper and the very complete data of Gayon and Dubourg and other investigators, it is suggested that the general line of reaction by which fructose is broken down and mannitol produced may be represented by the following equations.

$$\underset{\text{Fructose.}}{C_6H_{12}O_6} + H_2O = \underset{\text{Acetic.}}{C_2H_4O_2} + \underset{\text{Malic.}}{C_4H_6O_5} + 2H_2$$

$$\underset{\text{Malic.}}{C_4H_6O_5} = \underset{\text{Lactic.}}{C_3H_6O_3} + CO_2$$

$$2\underset{\text{Fructose.}}{C_6H_{12}O_6} + 2H_2 = \underset{\text{Mannitol.}}{2C_6H_{14}O_6}$$

If in the production of acetic and lactic acids from fructose nascent hydrogen is produced, this may then react with another fructose molecule and reduce it to mannitol. This theory does not regard mannitol as an intermediate but a coincident product in the formation of acetic and lactic acids. There is considerable evidence to support this theory in the papers of Gayon and Dubourg, Laborde, and Müller-Thurgau and Osterwalder. Carbon dixoide was always formed by their bacteria and malic acid was fermented to lactic acid and carbon dioxide by some of these organisms. Mannitol was found in quantities approximating what is required by this theory. Their organisms did not ferment mannitol and hence the theoretical amount of mannitol might accumulate. The data of Gayon and Dubourg, and Laborde are sufficiently complete to balance against that required by the above theory.

	Found.		Required.
	Gayon and Dubourg.	Laborde.	
	per cent	*per cent*	*per cent*
Acetic acid	14.0	10.0	11.1
Lactic "	12.5	14.3	16.7
Mannitol	66.0	63.0	67.4
Carbon dioxide	9.0		8.2
Succinic acid	0.25		
Glycerol	0.60		

When we consider the difficulties and errors involved in the determination of some of these compounds, especially lactic acid, for which all methods probably give too low results, it is seen that fair agreement exists between the found and calculated values. The small amounts of glycerol and succinic acid found could also be accounted for as originating from the malic acid.

That the above theory does not seem to fit all the facts in respect to each mannitol-producing organism that has been isolated will be observed by a careful examination of the published data; but as a working hypothesis it may well serve for a time. The correlation of the above theory with the data obtained in the fermentation of fructose by the pentose fermenters will be considered in the experimental part of this paper.

EXPERIMENTAL.

In the fermentation of fructose, approximately 2 per cent solutions were used. The sugar was dissolved in a water extract of compressed yeast sterilized for 30 minutes and sterilized bromocresol purple added at the time of inoculation. The acids formed during the fermentation were neutralized with sterilized normal alkali whenever a decided acid reaction was indicated by the bromocresol purple. Controls, treated as above but not inoculated, were always run parallel with the fermenting culture solutions. The values obtained from the controls have been deducted in calculating the data given in the tables. At the end of the fermentation period the cultures were analyzed for acetic and lactic acids by the steam distillation and extraction methods employed in our previous work (1919). At the same time the

mannitol was determined either on an aliquot of the culture or, in a few cases, upon the ether-extracted residue. The procedure in general was that previously used by Gayon and Dubourg and others. The sample was first concentrated on the steam bath, filtered from any precipitate, and the filtrate evaporated to dryness on clean sand. This was then successively extracted with small portions of boiling 80 per cent alcohol until completely exhausted of mannitol. About ten extractions were usually made. In some of the later determinations an extraction apparatus similar in design to the Kutscher-Steudel extraction apparatus for non-volatile acids was used. The lower end of the tube was immersed in an oil bath kept at a temperature just below the boiling point of the alcohol. The extraction could thus be carried on continuously for any desired length of time. Usually the dried residue was extracted for about 10 hours. After the alcohol extract was filtered, it was concentrated to a thick syrup and set aside in the refrigerator for 1 or 2 days. Crystals usually formed over night, but in some cases crystallization was not complete until after 2 or 3 days. The crop of crystals was freed from the mother liquor by filtering through a hardened filter with the aid of suction, was washed with alcohol and finally with ether, then dried, and weighed. As thus obtained the mannitol crystals were usually snow-white. The mother liquor and washings were again concentrated to a thick syrup, placed in the refrigerator, and kept there until a second crop of crystals had separated out. Usually 2 or 3 days were required. Additional crystallizations were made until no more crystals formed even after standing in the ice box for 6 or 8 days. With the improved method of extraction, the recovery of a known quantity of mannitol from yeast water and the control solution was from 92 to 95 per cent.

The unfermented fructose in the culture was also determined but in no case did this exceed 0.1 gm. and in most of the cultures merely a trace of reducing sugar was found. As the cultures contained about 2 gm. of fructose when inoculated, it is clear that practically all the fructose had disappeared at this time.

The results of the analyses are given in Table I and disclose several interesting points:

The quantities of acetic acid and lactic acid are nearly equal, but in general with lactic slightly in excess of acetic.

The quantity of mannitol is greater in the young cultures than in the older ones. This is very clearly shown in the 22 and 51 day cultures of Nos. 41–11 and 118–8. These four cultures were made up from the same batch of medium and were grown under the same conditions so that the results obtained furnish a good basis for comparison. The mannitol has decreased

TABLE I.

Products Formed from the Fermentation of Fructose in 100 Cc. of Culture.

Time.	Culture No.	Weight of fructose.	Acetic acid.	Lactic acid.	Mannitol.	Ratio acetic: lactic.
days		*gm.*	*gm.*	*gm.*	*gm.*	
16	41–11	1.70	0.312	0.329	0.573	100 : 105
22	41–11	1.70	0.320	0.340	0.441	100 : 107
51	41–11	1.70	0.436	0.549	0.323	100 : 126
16	118–8	1.70	0.311	0.338	0.533	100 : 108
22	118–8	1.70	0.293	0.151	0.350*	
51	118–8	1.70	0.444	0.539	Trace.	100 : 125
31	41–11	1.70	0.360	0.333	0.443†	110 : 92
31	55–9	1.70	0.353	0.328	0.443†	100 : 93
31	69–19	1.70	0.288	0.347	0.443†	100 : 120
31	69–30	1.70	0.331	0.290	0.443†	100 : 90
31	118–5	1.70	0.312	0.347	0.443†	100 : 111
31	118–8	1.70	0.326	0.335	0.443†	100 : 102
20	55–9	1.70			0.629†	
20	69–19	1.70			0.719†	

* Low result is probably due to bone-blacking the alcohol solution before crystallizing.

† This figure was obtained by the analysis of the combined Cultures 41–11 to 118–8 inclusive.

markedly in both cultures, while the acetic and lactic acids have just as markedly increased. The fructose present in parallel cultures analyzed at the end of 22 days was less than 0.1 gm., so that the increase in the acids could not have come from unfermented fructose. It must therefore have originated from the fermentation of the mannitol. The mannitol is formed very early in the fermentation of the fructose and then is itself slowly attacked by the organisms. Further evidence on this point will be presented later.

The identification of mannitol is based principally upon its characteristic crystalline form and upon its melting point. Many determinations, made on the crystals isolated, gave figures ranging from 163–164°C. (uncorrected). A sample of commercial mannitol gave a value of 164°C. (uncorrected). The melting point of pure mannitol is given by various authorities as 166.05°C. (Braham). The crystalline appearance, melting point, and fermentability clearly establish the identity of the compound.

In the preceding experiments no attempt was made to determine the gaseous products involved. After some preliminary tests it was found that carbon dioxide was produced in considerable quantities. A number of experiments were therefore carried out in which the carbon dioxide was determined and a more complete accounting for the fructose fermented was obtained.

In these experiments the fermentation flask A (Fig. 1) was closed with a two-hole rubber stopper. Through one hole was passed a short glass tube B. The upper end was closed by means of a short piece of rubber tubing and a screw pinch-cock. The rubber tube was plugged with cotton and wrapped with tin-foil. When it became necessary to add alkali to the fermenting solution the tin-foil cap and cotton were removed, the pinch-cock was opened, and sterilized alkali run in from a sterilized, finely calibrated pipette.

Through the second hole in the rubber stopper a bent tube C made from a 25 cc. pipette was passed. The delivery end of this tube passed through a two-hole stopper into a small bottle D containing strong potassium hydroxide. A guard tube E containing soda lime was joined to the potassium hydroxide bottle to prevent absorption of carbon dioxide from the air. As a further precaution against leaks the rubber stoppers were coated with a mixture of beeswax and paraffin. This apparatus permitted the gas evolved in the fermentation flask to pass over into the bottle containing the potassium hydroxide where the carbon dioxide was absorbed and at the same time permitted the escape of the air contained in the bottle at the beginning of the fermentation. The bulb of the connecting tube prevented the potassium hydroxide from being sucked back into the fermentation flask, when the temperature changed or when fermentation ceased. At the end of the fermentation period, a small glass tube was passed through

B to the bottom of flask A and the greater part of the CO_2 remaining in flask A was aspirated over into D by means of a current of CO_2-free air. The carbon dioxide in the culture solution and in the potassium hydroxide was determined by means of Van

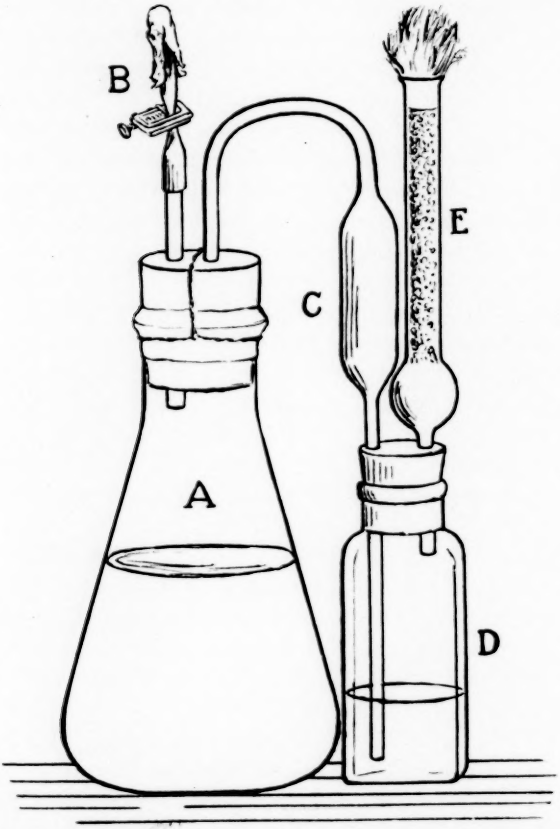

FIG. 1. Fermentation flask with CO_2 absorption bottle.

Slyke's apparatus for determining carbon dioxide in blood and carbonate solutions. The data obtained are given in Table II.

From the results of Table II it is seen that about 85 per cent of the fermented fructose is accounted for by the products acetic acid, lactic acid, mannitol, and carbon dioxide. The iodoform

test for alcohol was negative and the result by the potassium dichromate method for the quantitative determination of alcohol showed little more volatile acid than the reagents alone. The data obtained from the analyses of the *4 day* cultures of No. 118–8 bring out very clearly the rapidity with which fructose disappears from the culture solution; less than 0.1 gm. of fructose remained unfermented. About one-half the sugar consumed had been broken down into acetic and lactic acids and carbon dioxide. A somewhat smaller proportion of the sugar had been converted into the mannitol. The evidence indicates that the mannitol is not attacked until the more easily fermented fructose has been consumed. As will be shown later the slow rate of fermentation of mannitol lends support to this view.

TABLE II.

Total Fermentation Products Formed and Per Cent of Fructose Accounted for by the Products.

Time.	Culture No.	Weight of fructose fermented.	Acetic acid.	Lactic acid.	Mannitol.	Carbon dioxide.	Fructose accounted for by products.
days		*gm.*	*gm.*	*gm.*	*gm*	*gm.*	*per cent*
17	41–11	1.70	0.326	0.365	0.570	0.215	87
17	118–8	1.60	0.266	0.284	0.452	0.220	77
4	118–8	1.60	0.239	0.260	0.764	0.176	90
58	118–8	1.60	0.298	0.180	0.450	0.273	75

Due to the improved means of extraction already described, the values for mannitol obtained here are higher than in the earlier experiments.

From the data in Table II the molecular proportions of the several products have been calculated and appear in Table III.

The molecular ratios are in fair harmony with the reaction previously proposed.

$$\underset{\text{Fructose.}}{C_6H_{12}O_6} \rightarrow \underset{\text{Acetic acid.}}{CH_3COOH} + \underset{\text{Lactic acid.}}{CH_3CHOH.COOH} + CO_2$$

180	60	90	44
	1 mol.	1 mol.	1 mol.

The only serious departure is in the case of lactic acid. This is not unexpected since all the experimental errors tend to give a low value for lactic acid.

The possibility is always present that the lactic acid formed from the fructose may in turn be fermented subsequent to the fermentation of the fructose itself. This possibility is strongly indicated by the data obtained from the analysis of the 58 day culture. The data obtained from the fermentation of malic acid and old cultures of mannitol lend considerable support to this possibility. Kayser described a type of fermentation in which acetic acid and lactic acid were produced; but he believed that when fermentation was continued for some time the lactic acid was almost completely converted into acetic acid.

TABLE III.

Molecular Relations of the Fermentation Products of Fructose.

	Culture 41-11.			Culture 118-8.		
	Acetic acid.	Lactic acid.	CO_2	Acetic acid.	Lactic acid.	CO_2
Weights divided by molecular weights	0.051*	0.041	0.050	0.044*	0.031	0.050
Molecules	1.0	0.8	1.0	1.0	0.7	1.1
Weights divided by molecular weights				0.040†	0.029	0.040
Molecules				1.0	0.7	1.0
Weights divided by molecular weights				0.050‡	0.020	0.062
Molecules				1.0	0.4	1.5

* After 17 days fermentation.
† " 4 "
‡ Same culture as (†) analyzed 54 days later.

Fermentation of Malic Acid.

In order to test the hypothesis that malic acid may be an intermediate product in the fermentation of fructose by the pentose fermenters the fermentability of malic acid was demonstrated experimentally and the products formed were determined. In Series I a solution of malic acid was neutralized with sodium hydroxide and then made up with yeast water to a volume equivalent to 2 per cent calculated as malic acid. These solutions were inoculated with Cultures 41–11 and 118–8, and sterilized bromocresol purple was added. Fermentation of the sodium malate

was shown by the solutions becoming alkaline to the bromocresol purple. This change indicates the decarboxylation of the salt, the formation of sodium bicarbonate, and the production of a monobasic acid instead of a di-basic acid. The alkalinity thus developed soon checks the activity of the organisms. The cultures were therefore neutralized with 0.1 N malic acid at intervals of about 2 days each. That malic acid ferments very rapidly may be seen from the curve of acid production given in Fig. 2 at the end of this paper. The rate of acid production was somewhat slower at first than was the case with fructose, but soon became faster and continued at this very rapid rate to the end of the

TABLE IV.

Fermentation of Malic Acid Salts Calculated for 100 Cc. of Culture.

Culture No.	Length of fermentation.	Weight of malic acid.	Volatile acid as acetic.	Non-volatile acid as lactic.	Carbon dioxide.
	days	*gm.*	*gm.*	*gm.*	*gm.*
Series I.					
41–11	30	3.140*	0.146	0.804	Undetermined.
118–8	30	3.140*	0.176	0.734	"
Series II.					
41–11	16	0.684*	0.078	0.342	0.260
118–8	16	0.684*	0.070	0.268	0.216
Series III.					
41–11	23	2.000†	0.043	0.113	0.084
118–8	23	2.000†	0.059	0.111	0.102

* As the neutral sodium salt.
† " " " calcium "

fermentation period. The malic acid thus fulfills one of the requirements for an intermediate fermentation product; *viz.*, that the intermediate product must ferment as fast as or faster than the compound from which it is formed. The results of the malic fermentation are given in Table IV.

In Series II and III the carbon dioxide evolved was determined. Except in Series II, where only small amounts of malic acid were used, large amounts of malate remained unfermented. In Series I fermentation ceased because of the high alkalinity developed, caused by the inevitable accumulation of sodium bicarbonate. In Series III the alkalinity must be due to calcium

carbonate which is not injurious to the growth of the organisms. Some other factor must therefore have operated to check the fermentation in this case.

When the cultures were analyzed it was found that an appreciable amount of volatile acid had been developed. This acid was later identified by its Duclaux distilling constant as almost entirely acetic. The quantity of carbon dioxide found is greater than that required on the assumption that one molecule of carbon dioxide is formed for one molecule of lactic acid. The production of acetic acid and an excess of carbon dioxide, therefore, indicates that some change other than the simple malic to lactic takes place. The origin of the acetic acid and the excess of carbon dioxide might be explained on the assumption that a secondary fermentation of the lactic acid follows the primary malic to lactic fermentation. On this hypothesis the breaking down of the malic acid may be represented schematically.

$$\underset{\text{Malic acid.}}{COOH.CH_2CHOH.COOH} = \underset{\text{Lactic acid.}}{CH_3CHOH.COOH} + CO_2$$

$$\underset{\text{Lactic acid.}}{CH_3CHOH.COOH} \rightarrow \underset{\text{Acetic acid.}}{CH_3COOH} + CO_2$$

This is only a working hypothesis and is subject to more complete experimental data.

The possibility of organic acids other than malic being intermediate products in the fermentation of fructose was tested, but none was found that could be fermented. The malic acid cultures were tested for alcohol, but none was found.

Fermentation of Mannitol.

In the course of a study on the pentose fermenters it was found that mannitol was broken down with the formation of acetic and lactic acids. An experiment was therefore set up to test the fermentability of the mannitol, formed from fructose, by the same bacteria that produced it. For this purpose 40 gm. of fructose were dissolved in 2 liters of water and 100 cc. portions placed in twenty flasks. Ten flasks were inoculated with Culture 41–11 and ten with Culture 118–8. The mannitol produced from 30 of the 40 gm. of fructose was isolated as

previously described and amounted to 5.97 gm. Culture solutions containing 2 per cent of this mannitol were then inoculated with the six pentose fermenters previously studied and the acid production was roughly measured by the addition of normal alkali from time to time. After varying lengths of time the cultures were analyzed for acetic and lactic acids. The data obtained together with that from the fermentation of commercial mannitol are given in Table V.

TABLE V.

Acid Production from Fermentation of Cultures Containing 2 Per Cent Mannitol. Calculated for 100 Cc. of Culture.

Time.	Culture No.	Manitol used.	Volatile acid as acetic.	Non-volatile acid as lactic.	Ratio acetic: lactic.
days			*gm.*	*gm.*	
18	41–11	Merck's C.P.	0.185	0.221	100: 120
18	55–9	"	0.190	0.232	100: 123
18	69–19	"	0.146	0.230	100: 158
18	69–30	"	0.151	0.184	100: 122
18	118–5	"	0.155	0.175	100: 113
18	118–8	"	0.137	0.194	100: 142
66	41–11	"	0.560	0.788	100: 141
37	55–9	"	0.325	0.522	100: 160
66	69–19	"	0.317	0.497	100: 156
60	118–8	"	0.266	0.360	100: 135
42	118–8	From fructose.	0.415	0.499	100: 120
87	41–11	" "	0.798	0.306	100: 38
87	55–9	" "	0.544	0.228	100: 42
38	69–19	" "	0.352	0.507	100: 144
38	69–30	" "	0.239	0.305	100: 128
38	118–5	" "	0.224	0.282	100: 126

An examination of the data shows a slow but steady fermentation. After 18 days the acids formed represent about 20 per cent of the initial mannitol and at the end of 66 days this has risen to as high as 67 per cent in the case of Culture 41–11. Only slight differences in the vigor of the cultures are apparent during the early stages of the fermentation, but at the end more marked differences appear. Cultures 41–11, 55–9, and 69–19 show conspicuous activity in the fermentation of both mannitol and fructose. The ratio of acetic to lactic runs much the same throughout the period of fermentation except in the two 87 day

Cultures 41–11 and 55–9, where a very pronounced change in the ratio has taken place. Apparently in these two cases some of the lactic acid has been broken down into acetic acid. This possibility was also pointed out in connection with the fermentation of fructose, Tables II and III, malic acid, Table IV, and in the discussion of Kayser's work on the fermentation of glucose.

The commercial C. P. mannitol and the mannitol from fructose are fermented in exactly the same manner. The rate of fermentation, the products formed, and the ratio of the two acids are alike in both cases. Biologically, as well as chemically, the mannitol from fructose appears to be the same compound as the commercial product.

Identification of the Volatile Acids.

The volatile acids formed from the fermentation of fructose, mannitol produced from fructose, Merck's C. P. mannitol, and malic acid were subjected to a Duclaux distillation and their distilling constants determined. The data obtained in a few representative cases are given in Table VI.

It is evident from this table that the volatile acid formed by the fermentation of fructose mannitol, whether the commercial or biological product, and of malic acid is practically all acetic acid. The agreement between the distilling constants obtained and that required for acetic is very good. In a few representative cases the barium content of the acids used in the Duclaux distillation was determined subsequent to this distillation, and serves further to identify the volatile acid as nearly pure acetic. The data are given in Table VII.

Identification of the Non-Volatile Acid.

The non-volatile acid was identified as lactic acid by its barium salt and zinc salt. The free acid was extracted by the usual ether extraction method and titrated with barium hydroxide; from the barium salts the zinc salts were made. The zinc lactate crystals thus obtained had the crystalline appearance of the inactive or racemic type (Dox and Neidig). The determination of the water of crystallization showed that three molecules of water were present for each molecule of salt. The data obtained are given in Table VIII.

TABLE VI.

Distilling Constants of the Volatile Acids Obtained by Duclaux Method.

Source of acid.	Culture No.	Fractions.									
		10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
Fructose	41–11	7.1	15.8	24.3	33.1	42.2	51.8	62.0	73.0	85.2	100
"	118–8	7.1	16.4	24.8	33.5	42.6	52.1	62.3	73.3	85.5	100
Mannitol from fructose	69–19	7.5	15.5	24.0	32.7	41.9	51.6	62.0	73.0	85.0	100
Mannitol (Merck's C.P.)	41–11	7.4	15.3	24.0	32.2	41.6	51.3	61.5	72.5	85.2	100
Malic acid	41–11 and 118–8.	7.8	15.9	24.2	32.8	41.8	51.2	61.4	72.5	84.9	100
Duclaux constant for acetic acid		7.4	15.2	23.4	32.0	40.9	50.5	60.6	71.9	84.4	100

TABLE VII.

Composition of the Barium Salts of the Volatile Acid.

Compound fermented.	Culture No.	Barium salts of the volatile acid.	Barium sulfate equivalent.	
			Found.	Calculated for acetic acid.
		gm.	*gm.*	*gm.*
Fructose	118–8*	0.3854	0.3488	0.3521
"	118–8†	0.7566	0.6816	0.6913
Mannitol from fructose	41–11	0.3314	0.3030	0.3028
" (Merck's C. P.)	41–11	0.5748	0.5151	0.5234
Sodium malate	41–11 and 118–8.	0.3416	0.3048	0.3121

* Fermented 4 days.
† " 58 "

TABLE VIII.

Water of Crystallization Contained in Zinc Lactate.

Source of salt.	Culture No.	Zinc lactate used.	Water lost.		Water in $Zn(C_3H_5O_2)_2+3H_2O$.
		gm.	*gm.*	*per cent*	*per cent*
Fructose	118–8, 3 days old.	0.1472	0.0260	18.2	18.17
"	118–8, 12 days old.	0.1186	0.0208	17.6	18.17
Mannitol from fructose	69–19	0.3022	0.0602	19.1	18.17
" (Merck's C.P.)	41–11	0.464	0.0824	17.8	18.17

In a few cases the non-volatile acid was examined for its barium content. After the ether extract was titrated with barium hydroxide, the solution of barium salts was evaporated to about 10 cc., made up to 100 cc. with 95 per cent alcohol, filtered from any precipitate, and an aliquot evaporated to dryness in a platinum dish. The salts were dehydrated by heating at 130°C. for several hours until constant weight was secured and then were converted into barium sulfate by burning off the salts in the presence of an excess of sulfuric acid.

From the data thus obtained it is apparent that the non-volatile acid is almost wholly lactic acid. No evidence of succinic or other acids was obtained. The slightly lower values found are

TABLE IX.

Lactic Acid Produced from Fructose and Its Related Compounds.

Compound fermented.	Culture No.	Weight of barium salts.	Weight of barium sulfate.	
			Found.	Calculated.
		gm.	*gm.*	*gm.*
Fructose	41–11	0.1630	0.1176	0.1206
"	118–8	0.1640	0.1206	0.1214
Mannitol from fructose	118–5	0.3128	0.2238	0.2315
" (Merck's C.P.)	118–8	0.5466	0.3900	0.4044
Malic acid	41–11	0.5386	0.3912	0.3985
" "	118–8	0.4862	0.3564	0.3597

due to the presence of a small amount of soluble organic matter, which was produced in the sterilization of the sugar and was weighed together with the barium lactate. The data are given in Table IX.

Rate of Acid Production.

As measured by the production of acid, fructose is very readily fermented by the pentose fermenters. The rate of acid production is not so fast as in the case of xylose and arabinose, but much faster than from glucose or galactose. The acidity of the fermenting solution, as indicated by the bromocresol purple, was neutralized from time to time by the addition of normal alkali. In this way the rate of development of acidity was noted. In Table X

are given the cc. of 0.1 N alkali or acid added at different times to cultures of fructose, commercial mannitol, mannitol obtained from fructose, and malic acid salts. From fructose approximately 40 cc. of 0.1 N acid were produced within 3 days, while from mannitol less than one-half this amount was produced. The fermentation in the case of fructose starts off quickly and is conspicuously faster for 15 to 20 days than in the case of mannitol. At the end of 23 days the quantity of acid produced from the two materials is nearly equal.

TABLE X.

Rate of Acid Production from Fructose, Mannitol, and Sodium Malate. Total 0.1 N Acid in 100 Cc. of Culture.

Time	Fermentation of fructose.				Fermentation of Merck's mannitol.				Fermentation of mannitol from fructose.				Fermentation of sodium malate.	
	Culture No.				Culture No.				Culture No.				Culture No.	
	41–11	55–9	69–19	118–8	41–11	55–9	69–19	118–8	41–11	55–9	69–19	118–8	41–11	118–8
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
3	40	36	31	40	25	12	5	9	6	7	10	6	10	10
6	65	65	53	67	58	27	31	22	22	23	26	20	50	50
10	80	80	68	76	70	45	51	37	37	40	42	30	90	90
18	90	88	80	85	85	69	64	53	61	73	63	53	145	145
27	103	101	92	96	106	91	75	66	82	102	84	75	180	180
38									100	128	106	100		
51									124	136				
63									147	142				

The conversion of fructose into mannitol is much more rapid than the breaking up of the latter into acetic and lactic acids and hence results in the accumulation of the mannitol. It is possible that no mannitol is attacked until most of the fructose has been used, because the latter compound is more readily utilized than the former. If the formation of mannitol is merely incidental to the strong reducing conditions set up in the fermentation of the fructose, then the mannitol would probably not be attacked until all the fructose had been consumed.

The rate of fermentation of the sodium malate, as judged from the production of acid, is equal to that of the fructose itself, and continues at this high rate throughout the fermentation period.

From the data in Table X four composite curves have been constructed to represent the average rate of acid production from the compounds fermented. The average of the figures for all the cultures at any given time has been taken to represent the acid produced at that particular time from each of the above four types of fermentation. Examination of Fig. 2 shows the general progress of the fermentation.

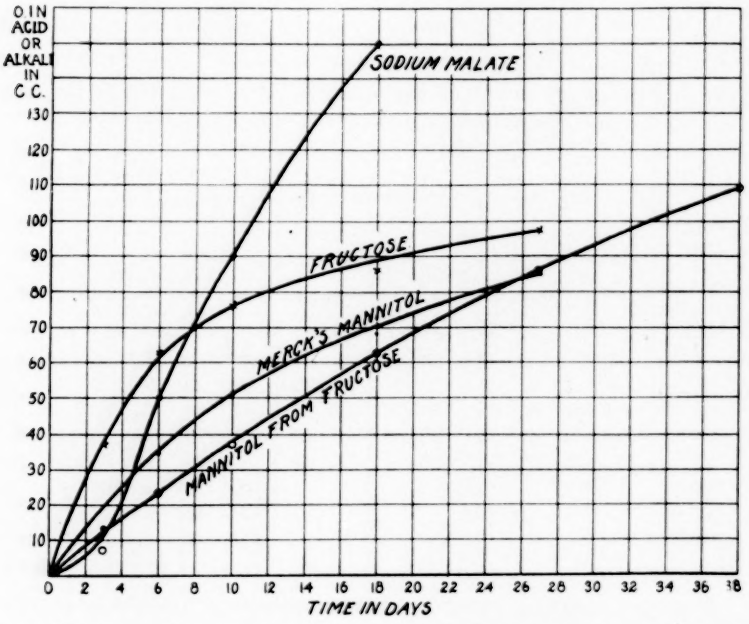

FIG. 2. Curves show average rate of acid production from different substances.

SUMMARY.

1. Fructose is readily fermented by *Lactobacillus pentoaceticus*, *n. sp.*, with the production of acetic acid, lactic acid, mannitol, and carbon dioxide.

2. The mannitol accumulates in considerable quantities, representing 30 to 40 per cent of the fructose in the early stages of the fermentation. It has the crystalline appearance and the same

melting point, 164°C. (uncorrected), as the commercial C.P. product.

3. With the mannitol thus obtained a culture medium can be made and fermented by the same bacteria that produced the mannitol. The products formed are acetic acid and lactic acid. The fermentation of this mannitol is identical with that of commercial mannitol both with respect to the rate of fermentation and the end-products formed.

4. If the fructose solution is allowed to ferment for a long time the mannitol, which accumulates at first, undergoes slow fermentation; acetic and lactic acids are produced and eventually all the mannitol is destroyed.

5. The production of acetic and lactic acids from fructose is very rapid during the first days of the fermentation but slows up after 10 days. All the fructose disappears within 5 days. The fermentation of mannitol, on the other hand, is slow in starting but continues at about the same rate throughout the entire period. This difference in the fermentation of the two compounds is explained by assuming that the strong reducing conditions set up by the rapid production of acetic and lactic acids quickly convert the unfermented fructose into mannitol. Within a few days the fructose has thus been used up in the production of acetic acid, lactic acid, carbon dioxide, and mannitol. The slower fermentation which then ensues is really the decomposition of mannitol.

6. Sodium and calcium malate are fermented by the same organisms and yield chiefly lactic acid and carbon dioxide and a small quantity of acetic acid as the end-products.

7. On the basis of the above facts it is suggested that the main line of the fermentation process is the production of acetic acid, lactic acid, and carbon dioxide. It is assumed that malic acid is an intermediate compound in this process. The strong reducing conditions incident to this fermentation result in the reduction of a large part of the unfermented fructose to mannitol. This mannitol is subsequently attacked; and thus a second fermentation entirely different from that of fructose is set up.

8. Some evidence is presented to indicate that in a fermentation extending over a long period lactic acid itself is broken down to acetic acid by the pentose-fermenting bacteria.

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NUTRITIVE FACTORS IN PLANT TISSUES.*

III. FURTHER OBSERVATION ON THE DISTRIBUTION OF WATER-SOLUBLE VITAMINE.

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In continuation of our earlier efforts to secure a more accurate comparison of the content of water-soluble vitamine of natural foods[1] the following method was employed with a series of plant products. Alfalfa, cabbage, clover, spinach, and timothy were dried in the manner already described.[1] Beet, carrot, and turnip roots were washed, cut into thin slices, desiccated in an air drier at 50–60°, and ground finely. Commercially canned tomatoes were dried at 60–70° and ground. Whole potato consisted of unpeeled potatoes boiled until tender, drained, mashed or sliced, dried at 60–70°, and ground. Peeled potato was prepared by paring raw potatoes as in domestic practise, boiling them until tender, mashing without draining, drying at 60–70°, and grinding. Furthermore, to compare the parts of the entire potato tuber with respect to the distribution of the water-soluble vitamine the potato peel was boiled in distilled water until tender, dried without draining, and ground.

These dried products were fed, apart from the rest of the food, to growing white rats receiving a diet adequate for them except

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington.

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187; xxxix, 29.

in respect to the lack of water-soluble vitamine. The basal food mixture consisted of:

	per cent
Meat residue	19.6
Salt mixture*	4.0
Starch	52.4
Butter fat	9.0
Lard	15.0

* For composition of salt mixture see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

The weekly food intake was determined except in a few instances where the animals spilled their ration and made the estimation inaccurate.

There are two ready methods of testing products for the presence of water-soluble vitamine. One consists in feeding the material to be investigated to animals that have declined on a diet deficient in this food factor. Such tests, however, will not demonstrate the *comparative* vitamine content of different foods. They are essentially restorative in character. If the outcome is positive in the sense of a renewal of nutritive well being, the test for the vitamine may be qualitatively successful. However, animals which have suffered a vitamine deficiency may be so badly malnourished or underfed that relatively excessive amounts of the vitamine-bearing food may be required to restore them to normal condition. In that event a failure to promote nutrition by the extract to be tested may be ascribed to the condition of the animal rather than the product. Comparisons of vitamine-bearing foods may not be reliable when they involve attempts to restore nutrition in animals that have suffered to a variable degree from unlike deficiencies.

The other method consists in feeding the supposed source of vitamine to animals which are normal in health and development, and observing whether the product offered supplies what is needed to promote normal growth when the diet affords an adequate supply of all essentials except that to be investigated. This procedure has been adopted in the present experiments. The basal ration included fat-soluble vitamine in the form of butter fat (9 per cent), but was devoid of water-soluble vitamine. Meat resi-

due, prepared in the manner already described by us[2] was selected as the source of protein because our earlier experiments showed this product to be practically free from the vitamine which it was here proposed to investigate. On the basal ration selected, growing rats of 60 to 120 gm. in body weight begin to decline in weight within a few days. Their food intake decreases and they invariably die within 80 days unless a source of water-soluble vitamine is furnished.[3] We fed the dried vegetable to be tested apart from the basal ration, so that the dosage might be accurately ascertained. With few exceptions the rats ate all the dried products each day; the data for this factor consequently have a strictly quantitative value in the comparisons between different tests.

Starting with healthy growing rats approximating 90 gm. in body weight the experiments were extended over 8 weeks with each dried food. The daily doses of the latter were varied, 1 and 0.5 gm. portions—in some cases 0.2 and 2 gm. doses—being tested.[4] At the end of this period, especially when growth had not proceeded at the normal rate, many of the animals were tested for a further period of 6 weeks with our standard mixed food or with daily additions of 0.2 gm. of dried brewery yeast[5] to determine whether the failures to grow satisfactorily had actually been due to lack of water-soluble vitamine rather than some other incidental factor.

The outcome of these comparative tests is shown in Charts I to X. In these experiments *alfalfa* and *clover* surpassed all the other nine products tested in equal doses as sole sources of water-soluble vitamine. Several of the animals (Rats 5769, 5768, 5775, 5790, Charts I and II) gained 175 gm. or more in weight during the 8 weeks period—a growth equal to the best which we have observed in our stock colony. This is a result far better than we

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 309.

[3] Osborne, T. B., Wakeman A. J., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxix, 35.

[4] The portions used were measured by means of small, calibrated scoops which were designed to deliver them in fairly accurate amounts. However, in the course of the experiments it was discovered that when the atmospheric humidity was unusually high the dosage of the dry powders furnished in this way was sometimes only approximately correct.

[5] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 149.

have ever secured with even 16 cc. of milk per day fed as a source of water-soluble vitamine in addition to the same basal ration. Half of this allowance, *i.e.* 0.5 gm. per day, of dry alfalfa or clover is not equally efficient (Rats 5744, 5750, 5723, 5772, 5770, 5774, Charts I and II).

Tomato is rich in water-soluble vitamine. 1 gm. daily doses promoted good growth when the animals would eat this quantity (Rats 5928, 6008, Chart III). At first it was frequently refused, perhaps because of the acrid flavor of the thus concentrated, highly acid product. 0.5 gm. doses of dried tomato were by no means so effective (Rats 5878, 5892, 5871), yet even 0.2 gm. quantities occasionally promoted limited growth or at least maintenance.

Spinach, *cabbage*, *turnip*, and *carrot* were not widely unlike in their comparative potency as sources of water-soluble vitamine. 1 gm. doses did not surpass 0.5 gm. quantities of alfalfa and clover (and perhaps tomato) in the results attained; whereas with the smaller amounts growth was not maintained (Charts IV to VII). The *beet root* (Rats 5972, 5980, Chart VI) did not equal the other roots tested. This is in harmony with the outcome of the observations of Steenbock and Gross[6] on the sugar beet and mangels, in contrast with the carrot, rutabaga, and dasheen. The *timothy* hay though potent in the earlier periods of the experiment (Rats 5931, 5867, 5865, Chart VIII) proved disappointing in the long run, in view of the expectations raised by the brief trials already reported.[7]

The *potato* is evidently as rich in water-soluble vitamine as some of the roots tested, although similar quantities of the leguminous hays, alfalfa and clover, or the tomato promoted more rapid growth. Compared in 1 gm. daily doses there was little if any advantage in potato retaining the outer layers (Rats 5954, 6021, 6017, Chart IX), over peeled potato of the same age (Rats 5961, 5955, 5978, Chart X). This corresponds with the observation that the dried *potato peel* (Rats 5963, 6037) is no richer in the vitamine than are corresponding quantities of whole potato (Rats 5926, 5975, 5977, 6042). Even 2 gm. daily doses of *peeled potato*

[6] Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1919, xl, 501.

[7] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxix, 29, Chart II.

(Rats 5981, 5984, 5985) did not promote growth at the normal rate during the entire 8 weeks period.

Hess and Unger[8] reported a slight difference in the antiscorbutic properties of old and fresh carrots. The comparative tests which we have made with *old* and *new* potatoes respectively in both 1 gm. and 0.5 gm. daily additions have not given indications of any noteworthy differences in content of the water-soluble vitamine here under investigation (Rats 5954, 6021, 6017, 5926, old potato; Rats 6082, 6044, 6086, 5975, 5977, 6042, new potato).

Bachmann[9] has reported that certain yeasts will not grow on synthetic media unless organic material is added in small amounts. The substances which when added to Nägeli's solution made it possible for the yeast to cause fermentation were said to be such as "have been found to be rich in vitamines, especially water-soluble B." Extracts were prepared from potato, carrot, celery, beet, onion, rutabaga, cabbage, cauliflower, parsnip, and lettuce. "All the extracts furnished the necessary substance for the yeast to grow and ferment the medium even though the amount added was very little . . . . the extracts were not equally potent." It is suggested by Bachmann that the method might be used to determine the presence of certain vitamines and even to determine quantitatively the amount present in various substances.

The statistical facts—total gains in weight and total food intakes in the 8 weeks period during which the dried plant products were fed by us in comparable doses each day to rats—are summarized in Table I.

As might be expected the greatest gains were in general the outcome of food intakes large in comparison with those of the rats which made smaller increments of weight. This is conspicuous in comparing the food intake and gains after feeding different amounts of the sources of vitamine. It should be noted that the figures recorded in the table as "gain" represent the end result of 8 weeks tests in the course of which a number of the rats, as shown in the charts of growth, reached larger size and subsequently declined somewhat owing, presumably, to the inadequate supply of vitamine. If the food intakes in cases of the smaller gains seem

[8] Hess, A. F., and Unger, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1919, xvi, 52.

[9] Bachmann, F. M., *J. Biol. Chem.*, 1919, xxxix, 235.

TABLE I.

*Total Gains in Body Weight and Food Intake.**
8 Weeks Period.

Food.	1 gm. daily.			0.5 gm. daily.		
	Rat No.	Gain in weight.	Total food intake.	Rat No.	Gain in weight.	Total food intake.
		gm.	*gm.*		*gm.*	*gm.*
Alfalfa.	5769	200	655	5744	144	536
	5768	179	594	5750	121	499
	5868	146	562	5723	106	516
Clover.	5775	197	663	5774	112	489
	5790	177	565	5770	102	441
	5791	150	497	5772	99	448†
Tomato.	6008	122	543	5878	88	453
	5936	120	516	5892	70	484
	5932	118	468			
	5928	115	544			•
Spinach.	5870	119	476†	5808	87	439
	6014	101	445	5817	61	364
	5767	81	395	5857	32	326
	5914	75	456			
Cabbage.	5907	99	472	5922	52	314
	5921	91	364	5918	49	314
	6009	58	472	5919	30	271
Turnip.	5920	96	406	5842	46	306
	5869	68	268	5895	30	312
	5820	66	347†	5839	20	258
Carrot.	5824	78	398	5819	49	334
	5816	78	439†	5805	38	306
	6016	66	393	5802	36	287
	5758	46	398			
Timothy.	5867	42	367	5912	22	261
	5931	40	342	5970	5	317†
	5865	10	314	5909	−2	214

TABLE I—*Concluded.*

Food.	1 gm. daily.			0.5 gm. daily.		
	Rat No.	Gain in weight.	Total food intake.	Rat No.	Gain in weight.	Total food intake.
		gm.	*gm.*		*gm.*	*gm.*
Peeled potato.	5961	63	355			
	5978	52	367			
	5955	45	358			
Whole " old.	6021	60	439	5926	11	269
	5954	57	351			
	6017	40	368			
" " new.	6086	83	366	5977	25	296
	6082	54	361	5975	5	306
	6044	43	362	6042	4	288
Potato peel.				6037	4	225
				5963	4	267
Beet root.	5980	6	240			
	5972	−9	246			
	0.2 gm. daily.					
Tomato.	5862	51	321			
	6018	32	347			
	6023	30	305			
	5866	2	255			

* The food intake is exclusive of the dried vegetable supplied.

† Some of the food was spilled by the rat so that the data are not entirely accurate.

disproportionately large it must be recalled that even when no growth whatever occurs a maintenance requirement must be met. In comparing the quota of the food devoted to growth, the maintenance requirement ought strictly to be subtracted from the total intake, and comparisons made on the basis of the difference. Inasmuch as the maintenance needs vary at different ages this calculation cannot be satisfactorily carried out with the data available. In earlier experiments with foods of similar make-up

we[10] found male rats, beginning at 90 gm. in weight, to make average gains of approximately 85 gm. in 8 weeks on a food intake of about 450 gm. Many of the animals in the present series have given even better results.

Inasmuch as the daily allowance of water-soluble vitamine was fixed, whereas the food intake varied with the individual and still more with the character of the plant product fed, the ratio of the latter to the total food eaten varied. In the case of rats receiving 1 gm. portions the vitamine-bearing vegetable product ranged from 10 per cent of the food intake or less in those which grew best, to 15 per cent or more at the other extreme of the series. In the 0.5 gm. experiments the content of plant food in relation to the total food ranged from about 5 to 9 per cent. Thus, the growth during the period when the dried tomato fed was equivalent to about 5 per cent of the food was greater than that in animals for which carrots supplied nearly three times this proportion. Dried brewery yeast is effective in far smaller proportions.[5] The method is not accurate enough and the statistics not sufficiently abundant to permit more than the approximate comparisons already referred to.

EXPLANATION OF CHARTS.

The following charts represent the growth of rats beginning with a body weight of approximately 90 gm. during a period of 8 weeks in which the sole source of water-soluble vitamine was supplied by the indicated quantities of various dried vegetable tissues, fed daily apart from an otherwise adequate standard food mixture. At the end of the 8 weeks feeding trial the growth capacity of the animals was further tested by feeding 0.2 gm. of dried brewery yeast in place of the dried vegetable, or, in a few cases, substituting the ordinary mixed food of our stock colony for the previous diet. The duration of the yeast feeding is indicated by the interrupted line (----------) and the mixed food by the designation (—•—·—·—). Days on which animals failed to eat all the dried vegetables offered are indicated by asterisks on the curve. The statistics for food intake, etc. are summarized in Table I.

[10] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351.

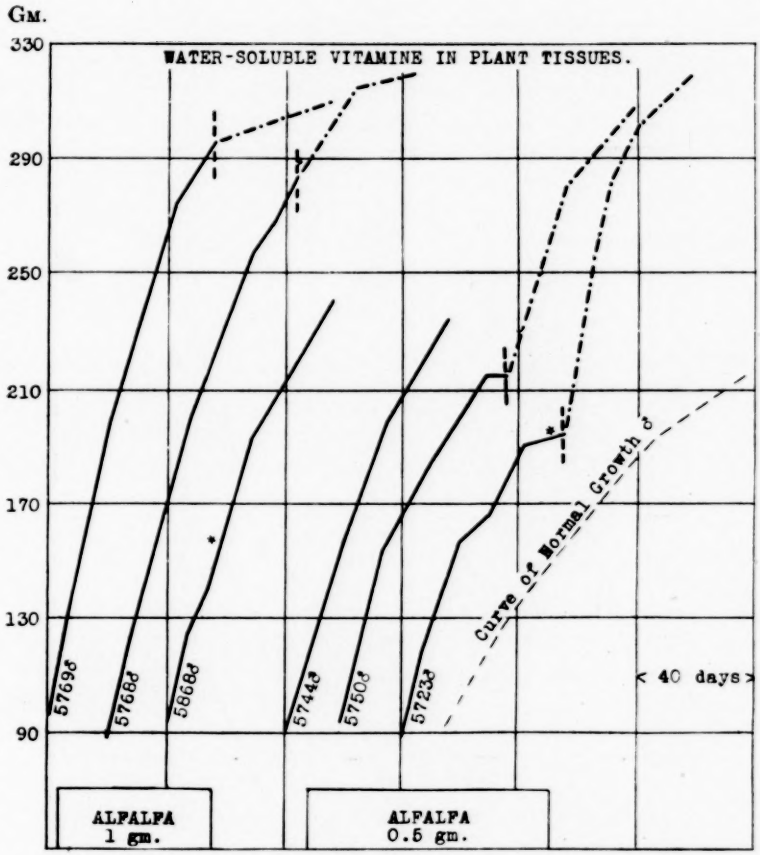
Gm.
330
290
250
210
170
130
90
WATER-SOLUBLE VITAMINE IN PLANT TISSUES.
Curve of Normal Growth ♂
< 40 days >
5769♂
5766♂
5868♂
5744♂
5760♂
5723♂
ALFALFA
1 gm.
ALFALFA
0.5 gm.

Chart I.

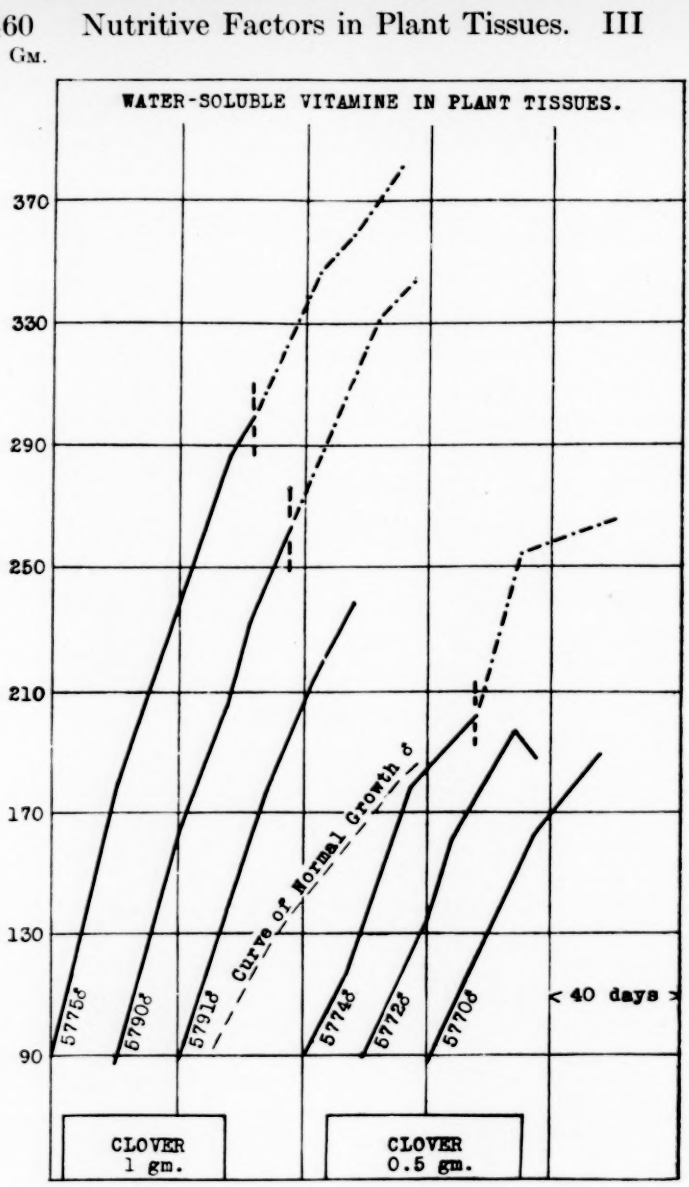
Gm.
WATER-SOLUBLE VITAMINE IN PLANT TISSUES.
370
330
290
250
210
170
130
90
5775♂
6790♂
5791♂ Curve of Normal Growth ♂
5774♂
6772♂
5770♂
< 40 days >
CLOVER
1 gm.
CLOVER
0.5 gm.

Chart II.

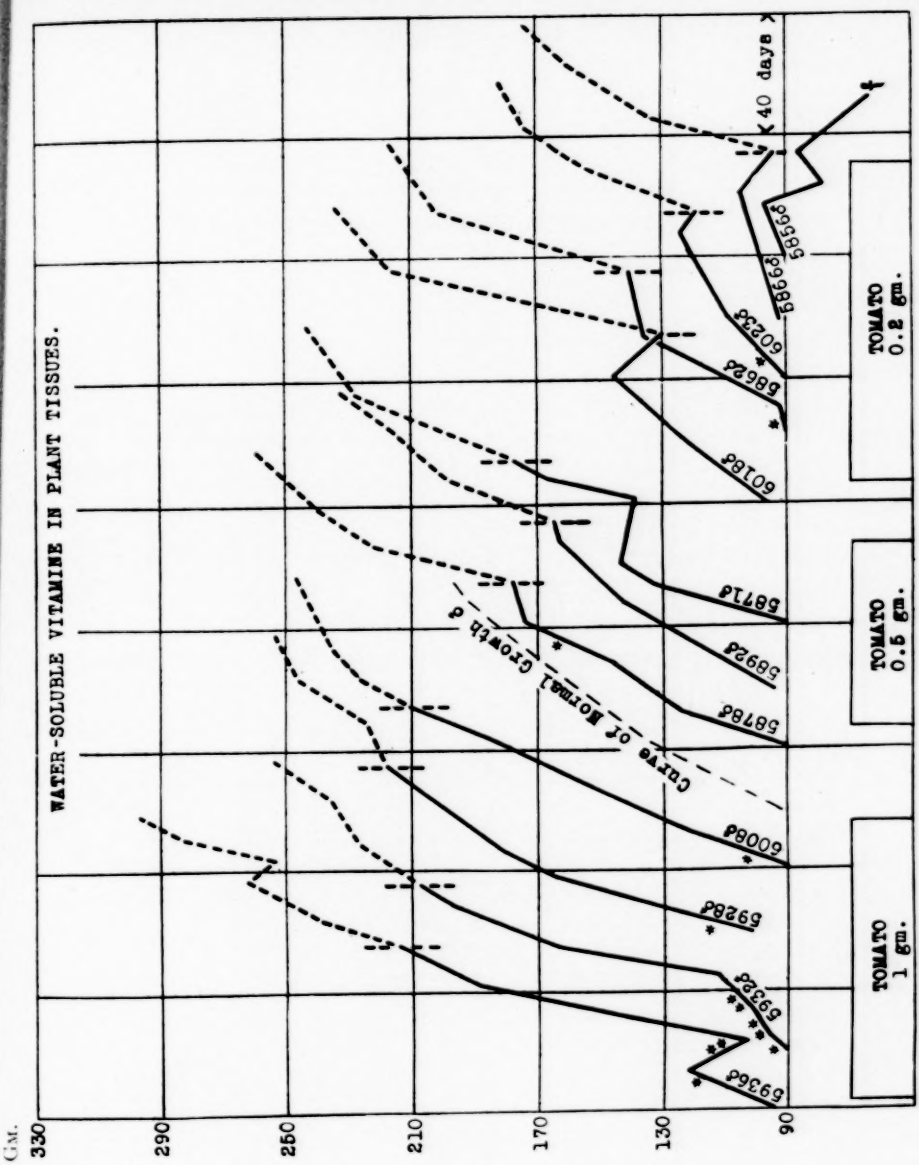
WATER-SOLUBLE VITAMINE IN PLANT TISSUES.
Gm.
330
290
250
210
170
130
90
40 days
Curve of Normal Growth
TOMATO 1 gm.
TOMATO 0.5 gm.
TOMATO 0.2 gm.
59368
59328
59288
60088
60088
60238
58628
60188
58718
58928
58788
60088
59288
59328
59368

Chart III.

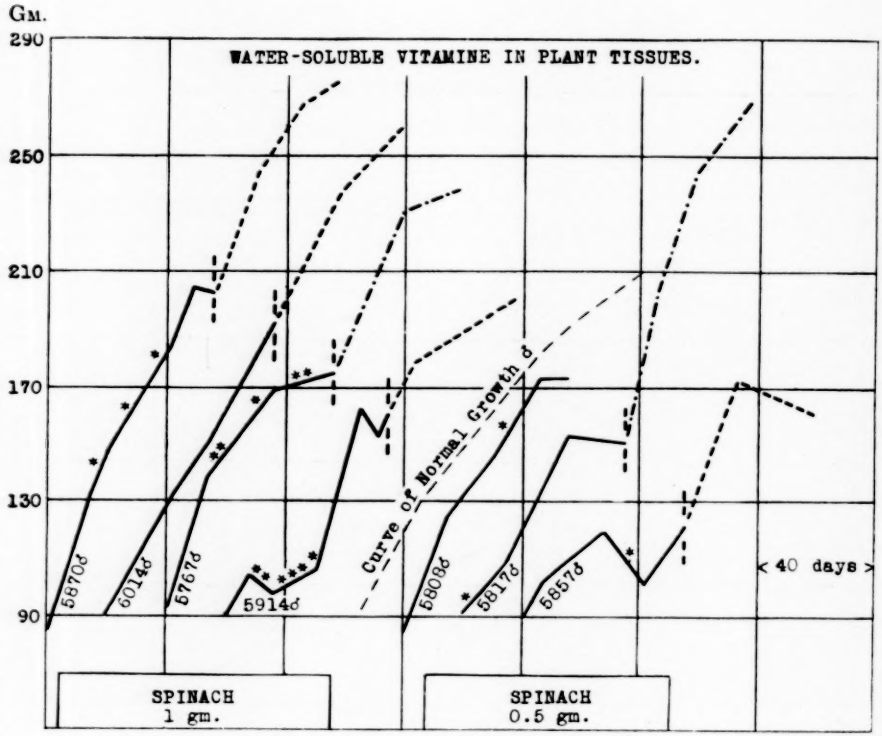
Gm.
290
250
210
170
130
90
WATER-SOLUBLE VITAMINE IN PLANT TISSUES.
5870♂
6014♂
6767♂
5914♂
Curve of Normal Growth ♂
5808♂
5817♂
5857♂
< 40 days >
SPINACH
1 gm.
SPINACH
0.5 gm.

Chart IV.

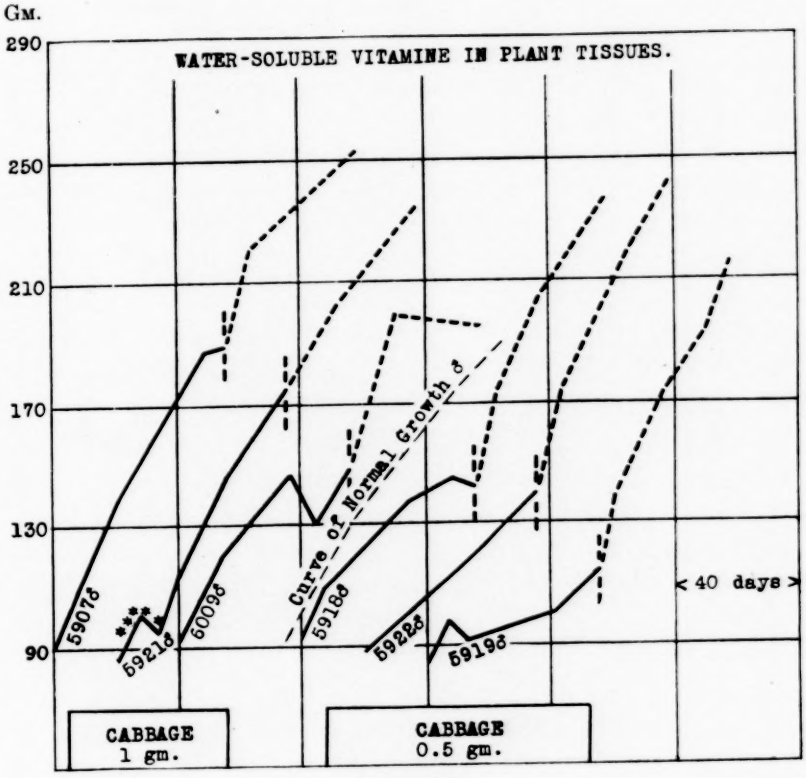
Gm.
290
250
210
170
130
90
WATER-SOLUBLE VITAMINE IN PLANT TISSUES.
Curve of Normal Growth ♂
59078
59218
60098
59188
59228
59198
< 40 days >
CABBAGE
1 gm.
CABBAGE
0.5 gm.

Chart V.

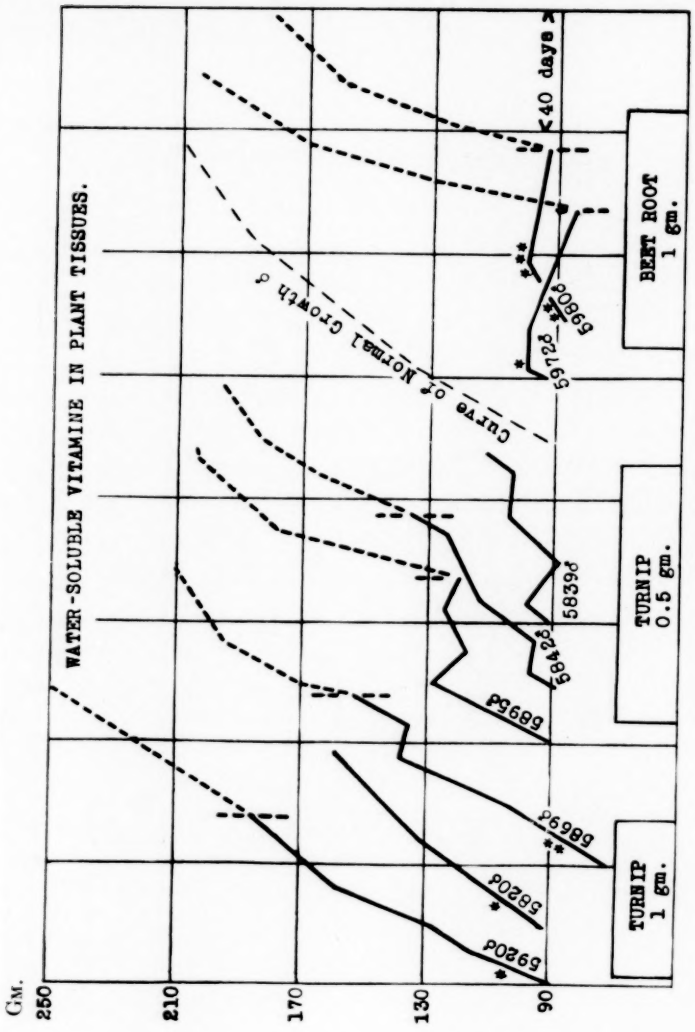
WATER-SOLUBLE VITAMINE IN PLANT TISSUES.
Gm.
250
210
170
130
90
Curve of Normal Growth δ
40 days
BEET ROOT 1 gm.
TURNIP 0.5 gm.
TURNIP 1 gm.
5920d
5820d
5869d
5895d
5842d
5839d
5972d
5980d

CHART VI.

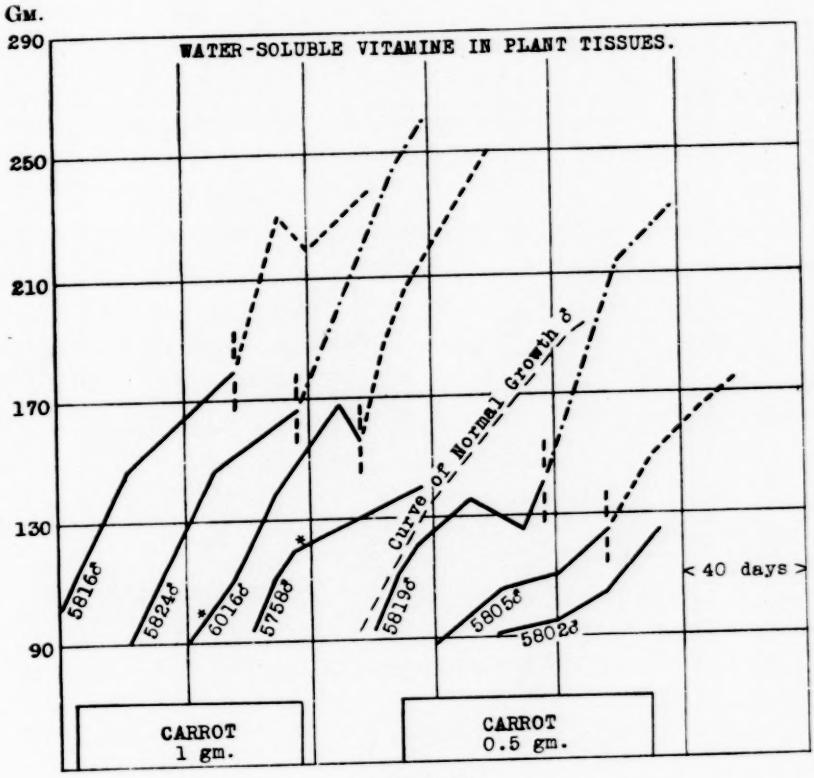
Gm.
290
250
210
170
130
90
WATER-SOLUBLE VITAMINE IN PLANT TISSUES.
Curve of Normal Growth ♂
5816♂
5824♂
6016♂
5758♂
5819♂
5805♂
5802♂
< 40 days >
CARROT
1 gm.
CARROT
0.5 gm.

Chart VII.

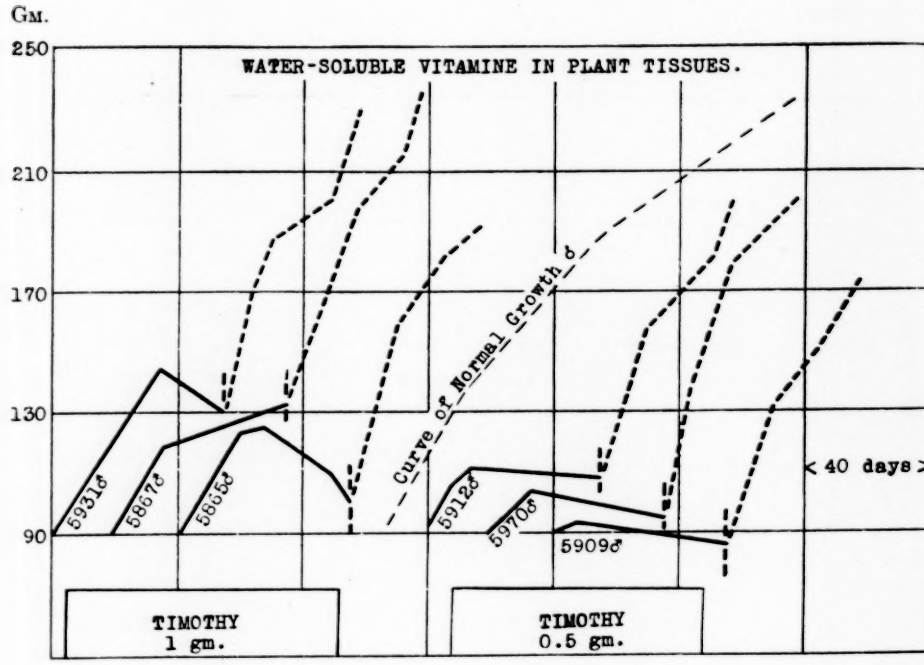
Gm.
250
210
170
130
90
WATER-SOLUBLE VITAMINE IN PLANT TISSUES.
Curve of Normal Growth ♂
5931♂
5867♂
5855♂
5912♂
5970♂
5909♂
< 40 days >
TIMOTHY
1 gm.
TIMOTHY
0.5 gm.

Chart VIII.

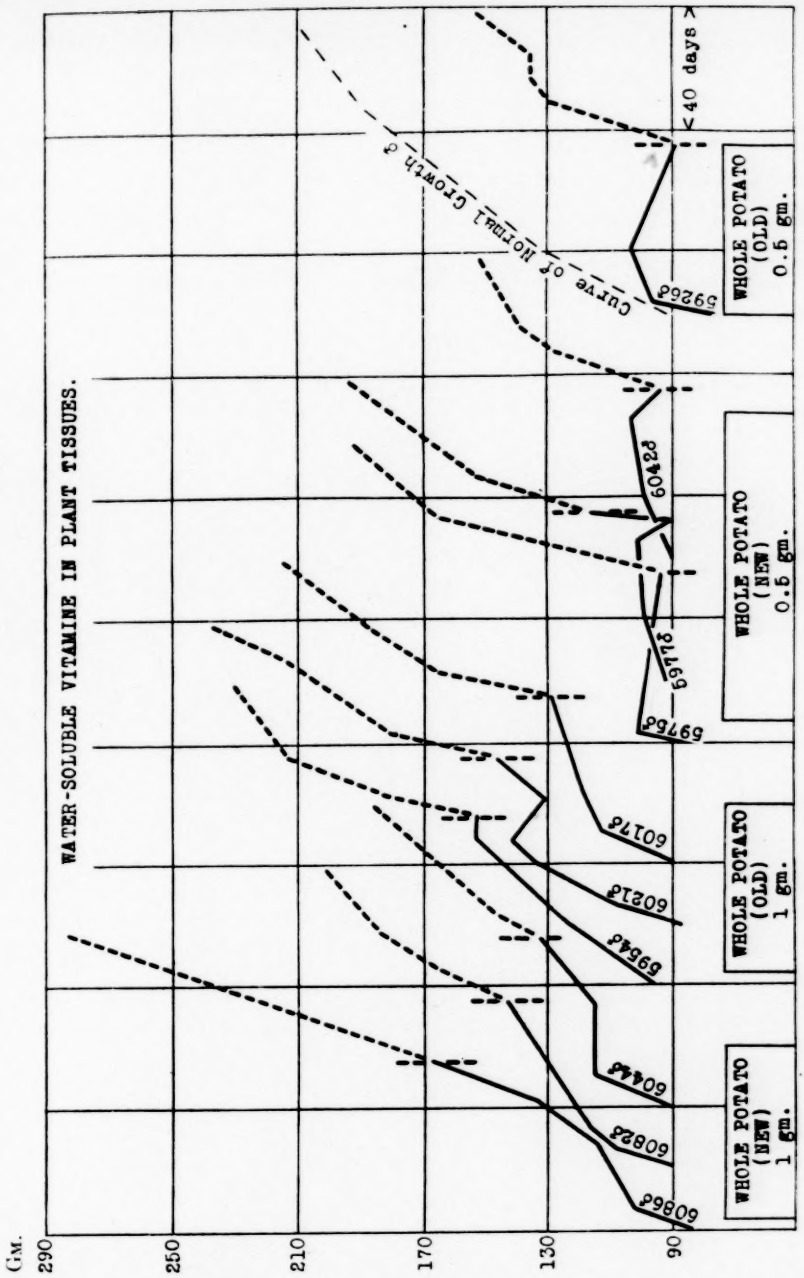
WATER-SOLUBLE VITAMINE IN PLANT TISSUES.
Gm.
290
250
210
170
130
90
Curve of normal Growth
< 40 days >
WHOLE POTATO (NEW) 1 gm.
WHOLE POTATO (OLD) 1 gm.
WHOLE POTATO (NEW) 0.6 gm.
WHOLE POTATO (OLD) 0.6 gm.
60866
60823
60443
59543
60213
60173
59763
59713
60263
59263

CHART IX.